Responses to Reflection in Two Invertebrate Species

by

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This thesis is dedicated to Susan Muriel Raymond, who planted the seed that began this journey. May she rest in peace.

ABSTRACT

The present thesis investigates the responses to reflection in both the crayfish *Procambarus clarkii* and the fruit fly *Drosophila melanogaster*. Responses to reflection in crayfish depend on social status and the current work suggests that learning and memory consolidation are required for these responses to be altered. Crayfish were treated to either massed or spaced training fights prior to reflection testing. The results show that subordinate crayfish treated to spaced training display a response typical of subordinate crayfish but subordinate crayfish treated to massed training exhibit a response typical of dominant crayfish. Fruit flies are shown to be attracted to reflection and responses to reflection are described here for the first time. Responses in fruit flies are shown to be dependent on social status. The frequency of behaviours were altered in isolated flies but not socialized flies. The addition of pheromones cVA and 7,11-HD were used to investigate how the addition of chemical cues altered responses to reflection in fruit flies. Socialized fruit flies treated with cVA exhibited an increase in the frequency of behaviours on both mirrored and clear glass walls, while isolated flies exhibited a decrease. Socialized flies treated with 7,11-HD spent more time on mirrored walls compared to glass walls, whereas the frequency of all behaviours were decreased in isolated flies treated with 7,11-HD.

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CHAPTER 1:

INTRODUCTION

1.01: GENERAL INTRODUCTION

This thesis examines responses to reflection in two invertebrate species, crayfish and fruit flies. Using mirrors to present a reflective environment (a mirror image) to an animal does more than simply provide a means to examine responsiveness to visual cues. Responses to reflected images can indicate whether or not the animal recognizes itself, and can demonstrate a high level of cognition associated with self-awareness as reported in a limited number of animal species. Other animals may perceive the image as a conspecific, indicating that they can perceive the image as another animal of the same species but they lack the ability to identify visual cues as an indication of "self". Still, other animals can look at a mirror image and not recognize it as anything other than an object, or a combination of moving shapes and colours. To investigate these scenarios, one must first show that the animal is attracted to the reflection, and then characterize the responses that ensue. The types of responses will indicate how the mirror image is perceived and from there we can learn what factors influence these responses.

This thesis provides evidence that responses to reflection in invertebrates are shaped by prior experience. Experiments performed with crayfish have already shown that dominance rank, which is established by winning or losing aggressive encounters with conspecific animals, alters the responses of crayfish to reflection, increasing the tendency of dominant crayfish to approach the reflection and increasing the tendency of subordinates to avoid it (May & Mercier, 2006; 2007). This thesis provides evidence that these changes in behaviour develop more rapidly when aggressive encounters follow a paradigm similar to those that produce learning and long-term memory, and it suggests that dominance ranks are learned and that the responses to reflection are indicative of

such learning. This thesis also shows, for the first time, that fruit flies are attracted to a reflective environment, and it identifies and characterizes their responses to their mirror image. The results suggest that the behaviours elicited by reflection in adult fruit flies are not related to aggression and are more likely to be related to courtship and/or mating. Furthermore, responses to reflection are compared between adult male flies reared in isolation and adult males reared socially, and the results demonstrate that social experience influences a fruit fly's responses to reflection. A possible interaction between responses to reflection and olfactory signalling is also examined in both socially naïve and socially experienced adult males, and results are presented here to indicate that *Drosophila* pheromones alter behaviours, but not as predicted.

Overall, the observations reported in this thesis, for both invertebrate species, indicate a key role for social experience in shaping the behaviours that are elicited and/or modified in response to the visual cues associated with a reflective environment. This seems appropriate in species that recognize a reflected image as a conspecific, since aggression-related responses should be appropriate for the subject's rank in the dominance hierarchy, and courtship-associated responses have been shown to be shaped by social experience. The following sections present a review of relevant literature describing how cues associated with different sensory modalities elicit and modify behaviour and how behaviour is shaped by social experience. These sections focus specifically on behaviours related to aggression, courtship and mating.

1.01.2: SENSORY CUES ELICIT BEHAVIOUR

Animal perception, or the ability to determine sensory input from one's environment, has been researched for more than 100 years. While more animals use all of the primary senses, that is, visual, tactile, auditory, taste and smell (chemosensory), the more prominent work in the field seems to focus on the examination of vision and what it can tell us about an animal's perception. With respect to invertebrates, a lot of researchers ask how visual cues aid in foraging or prey/predator detection. While chemosensory cues are used by insects such as wasps (D'Adamo & Lozada, 2003) and fruit flies (Frye et al., 2003) to detect the general area of food sources, visual cues are required to pinpoint exact locations. In fruit flies, almost half of the brain is devoted to the optic lobes, suggesting that visual cues are critical (Fischbach & Dittrich, 1989). The present thesis investigates how visual cues, specifically responses to mirror images, alter behaviour in fruit flies and crayfish.

Visual cues have been known to elicit aggression in various animals, both vertebrate and invertebrate. Threat displays are used by many animals during agonistic interactions with conspecifics. Gekkonid lizards use a combination of posture and movement in combination with the colours and patterning in their body to signal aggression and threat to conspecifics (Marcellini, 1977). A review of visual cues in fish reports that visual threat displays are used by many fish species including stickleback, beta, salmon, gobies, swordtails and cichlids (Rowland, 1999). The visual displays reported often involve fin displays that make the fish appear larger or body movements that produce distinct coloured or patterned displays. Male cuttlefish display a specific

body pattern termed Intense Zebra Display, which involves darkening of the face (Adamo & Hanlon, 1996). This face darkening correlates with fighting intensity and appears to signal aggressive motivation. Hermit crabs raise and spread the chelipeds and/or raise themselves up higher using the appendages to elicit a threat to a conspecific (Hazlett & Bossert, 1965). These threat displays were almost exclusively performed during conspecific interaction and always elicited a change of behaviour in the conspecific.

Crayfish also exhibit aggressive posturing. The meral spread, characterized as a raised body with the chelae up and spread wide, is performed during agonistic interactions with conspecifics and associated with aggression (Bruski & Dunham, 1987). A similar posture was described by Livingston et al., (1980) following the injection of serotonin (5-HT) in lobsters. More recently, this effect has since been shown to differ from a meral spread and is not associated with aggression (Tierney et al., 2001). It is thought that the meral spread is a visual threat directed toward the opponent; however, it has been shown to occur independent of light. There are no reports of crayfish performing a meral spread in response to reflection.

The experiments presented here isolate visual input with the use of mirrors, with the exception of the last chapter, which investigates the addition of pheromones to responses to reflection in fruit flies. Chemical cues play an important role in fruit flies. Fruit flies have sexually dimorphic pheromones that have uniquely different effects on male and female fruit flies. The most prominent female pheromone is a cuticular hydrocarbon named (Z,Z)-7,11-heptacosadiene (7,11-HD) (Jallon, 1984; Ferveur, 2010). 7,11-HD has been demonstrated to evoke courtship and mating in male fruit flies (Billeter et al., 2009). The predominant male pheromone is 11-cis-vaccenyl acetate (cVA) (Wang

et al., 2011). Both of these pheromones were used to investigate how chemical cues influence responses to reflection in the present thesis.

1.01.3: SOCIAL EXPERIENCE

Social experience has a profound influence on behaviour. One of the most extensively researched aspects of social experience is isolation. In monkeys, social isolation for the first six months of life results in a gross absence of social behaviour (Harlow et al., 1965), Monkeys demonstrated abnormal sexual, aggressive and maternal behaviours. They also suffered from what appeared to be depression, marked by the lack of activity and even refusal to eat. Monkeys isolated for six months after birth, later treated with gradual exposure to young monkeys to evoke a maternal instinct, recovered most of normal social behaviour (Harlow and Suomi, 1971). Alternatively, rats reared in social isolation exhibit hyperactivity, demonstrated by an increase in locomotion (Morgan, 1973) and decrease in exploration of objects in their environment (File, 1978). A similar effect is seen in invertebrate species.

Cockroaches reared in social isolation display a reduction in exploration and foraging (Lihoreau et al., 2009). A reduction in mating and social skills was also observed. In honey bees, a reduction in mushroom body growth was seen in bees reared in isolation compared to those reared in social conditions (Maleszka et al., 2009). Male fruit flies reared in isolation exhibited more courtship behaviours in the presence of a female, compared to socially reared fruit flies, and were more successful at copulation (Kim & Ehrman, 1998). Alternatively, male fruit flies reared in enriched conditions, that

is, larger chambers filled with visual stimuli, are more successful at mating when compared to males raised in typical vials (Dukas & Mooers, 2003). Fruit flies are unique in that they can be isolated at the pupal stage and onward, producing adult flies that have never seen, smelled or touched another fly. This "blank slate" is convenient to compare to socially reared flies when studying how social experience affects any type of behaviour.

To examine dominance hierarchies in crayfish, many researchers will socially isolate them prior to the experiment (Yeh et al., 1996, Zulandt Schneider et al., 2001, Bergman & Moore, 2005). This is often done to eliminate the effects of prior social rank, such as the "winner effect", where crayfish that win an agonistic encounter are more likely to win subsequent encounters, and the "loser effect", where crayfish that lose agonistic encounters are more likely to lose subsequent bout (Hsu & Wolf, 1999; Bergmann et al., 2003). Observations in previous experiments suggest that this social isolation depresses the behaviour of crayfish and that they are less likely to respond to their environment. Crayfish that were isolated prior to examining their responses to reflection exhibited no attraction or avoidance of the mirror image (May & Mercier, 2006).

Other social experiences, such as social status, are important in forming behaviour. Most social animals form dominance hierarchies, which aid in group dynamics and ultimately reduce aggression. Monkeys (Sapolsky, 2005), pigs (Fernandez et al., 1994), birds (Schneider, 1984; Lamprecht, 1986), dogs (Cafazzo et al., 2010) and fish (Fox et al., 1997) establish hierarchies of dominance. Invertebrate animals establish dominance hierarchies in much the same way. Insects that live in colonies rely on pheromones to distinguish between self and conspecifics (Ayasse & Paxton, 2002).

Agonistic interactions determine hierarchy order in bees (Bernasconi et al., 2000), wasps (Ruther et al., 2002) and ants (Beye et al., 1998). Of all invertebrates, aggression has been studied mostly in crustacea, such as lobsters, crab and crayfish (Kravitz & Huber, 2003). With subtle differences, all three of these crustaceans engage in paired fights that consist of striking and grasping with claws, pushing, flipping and antennae whips and flicks. These actions lead to one of the pair losing the fight by retreating from it. Losing animals eventually become subordinate while winning animals become dominant. In crabs, dominance status results in elevated levels of dopamine, serotonin and octopamine in the circulation (Sneddon et al., 2000). While all three of these biogenic amines play a role in aggression in lobsters, serotonin appears to have the foremost influence (Kravitz, 2000). While serotonin appears to play a role in aggression in crayfish, its role is more ambiguous (Yeh et al., 1996). Examination of the genetic control of aggression and social status has been limited because of the lack of gene studies in crustacea. For this reason, many researchers have moved on to other model systems to investigate the role of genes.

The fruit fly, *Drosophila melanogaster*, an insect species studied in the present thesis, also engages in agonistic contests to develop dominance hierarchies. Although this was reported in past studies (Dow & Schilcher, 1975; Hoffmann, 1987), pair-wise interactions were not expressly studied until recently (Chen et al., 2002). Such studies demonstrated that fruit flies exhibit a full complement of aggressive behaviours including boxing, lunging, tussling, chasing and wing threats. Recent genetic studies have shown that while serotonin affects the fly's ability to escalate aggression (Alekseyenko et al., 2010), the ability to exhibit aggression is controlled by single dopaminergic neurons (Alekseyenko et al., 2013).

1.01.4: RESPONSES TO REFLECTION

Responses to reflection have been examined in many species in the animal kingdom. Self recognition in a mirror has been demonstrated in four non-human primate species, including bonobos (Westergaard & Hyatt, 1994), chimpanzees (Gallup, 1970), orangutans (Suarez & Gallup, 1981), and has also been demonstrated in some non-primate vertebrates, such as elephants (Plotnik et al., 2006), dolphins (Reiss & Marino, 2001), killer whales (Delfour & Marten, 2001) and, most recently, the magpie (Prior et al., 2008). Most of these experiments involved some version of the Mark Test developed by Gallup in 1970. The Mark Test involves applying a mark on an area of the animal, usually the forehead, not visible to the animal itself. If the animal directs its attention to the mark when presented with a mirror, such behaviour demonstrates that the animal recognizes the mirror image as itself. This requires self-awareness, which was previously thought to occur only in human beings.

Animals that don't recognize themselves in the mirror often recognize the mirror image as a conspecific. This has been demonstrated in animals throughout the animal kingdom including non-human primates (Harris & Edwards, 2004), chicks (Montevecchi & Noel, 1978), cuttlefish (Palmer et al., 2006) and many fish species (Sovrano et al., 2001). Despite this, few invertebrate species have been studied for their responses to their mirror-image. Hermit crabs have been shown to perform appropriate social displays in response to their reflection (Dunham et al., 1986) and responses to reflection have been investigated by the present laboratory (Drozdz et al., 2006; May & Mercier, 2006; 2007). From our previous studies we know that the response to reflection in crayfish depends on their social status. Dominant crayfish are attracted to their mirror image and spend more

time in front of a mirror compared to a non-reflective environment when presented with a choice. Subordinate crayfish do not show a preference for reflection and in one experiment reverse walked more frequently in a reflective environment, suggesting avoidance behaviours (May & Mercier, 2006). These behaviours mimic responses to a conspecific and the data suggest that crayfish perceive their mirror image as a conspecific. We also know that these differences in responses take time to develop. While dominant crayfish exhibit a change in behaviour immediately following a 30 minute fight, subordinate crayfish must remain housed with the winning crayfish for at least three days before changes in behaviour are observed (May & Mercier, 2007).

The present thesis investigates how responses to reflection develop in crayfish and tests the hypothesis that learning is taking place. The correlation between responses to reflection and dominance status may be an indication of the extent to which subordinate crayfish have learned their rank and have acquiesced to their losing status. If responses of subordinates to reflection are an indication of their willingness to acquiesce to subordinate status, their responses to reflection should change more rapidly with a training paradigm that promotes learning. I predicted that if crayfish pairing periods are separated by periods of rest, thus providing time to consolidate memory, subordinate crayfish will learn their status more quickly and will respond differently to reflection compared to crayfish provided with one long pairing period.

To delve further into how responses to reflection develop and how visual cues can be altered, I also chose to investigate fruit flies. The fruit fly, *Drosophila melanogaster*, is an ideal model system because of its short life span, ease in rearing and the vast genetic tools available to this species. The hypothesis that fruit flies are attracted to their

reflection and that responses to reflection depend on socialization is examined here. Fruit flies are reared in total isolation, from pupae, and their behaviours compared to those of fruit flies reared socially. The responses to reflection are examined and the first ethogram for effects of reflection on any insect species is reported here. To further investigate how responses to reflection can be modified, pheromones are introduced. It is predicted that the addition of other sensory cues would increase the frequency and intensity of behaviours performed in response to reflection. Although the pheromones altered responses to reflection, the behavioural changes were not as predicted.

All of the preparation, experiments and analysis described in this thesis were performed solely by me, Holly May.

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CHAPTER 2:

RESPONSES TO REFLECTION IN SUBORDINATE CRAYFISH DEPEND ON LEARNING

2.01: ABSTRACT

Responses to reflection have previously been examined in socialized crayfish. The response has been shown to depend on dominance status. Dominance status is revealed by determining the outcome of fights that occur during pairing. This report demonstrates that agonistic contests between crayfish pairs are a training paradigm in which losing crayfish learn to become subordinate. Crayfish were exposed to three 30 min pairings, either presented in one trial (massed training) or three trials separated by 30 min intervals of rest (spaced training). Immediately following the training protocol, crayfish were placed into an aquarium where half of the walls were reflective while the other half were non-reflective, to examine their responses to reflection. Subordinate crayfish that were subjected to spaced training showed neither preference for reflection nor enhancement of reflection-dependent behaviours. This pattern of behaviour is equivalent to what is observed in subordinate crayfish paired for three days or more. Subordinate crayfish that experienced massed training behaved similar to dominant crayfish. These results show that losing crayfish learn to be subordinate more quickly following spaced training versus massed training.

2.02: INTRODUCTION

Crayfish are social crustacea that engage in agonistic behaviour when in the presence of a conspecific. This stereotyped behaviour leads to the formation and maintenance of a dominance hierarchy (Bovbjerg, 1953, 1956, Lowe, 1956). Hierarchies range from the most dominant (least submissive) to the least dominant (most submissive). Crayfish that win agonistic bouts rise in the hierarchy, while crayfish that lose will go down in rank. When crayfish are paired, the hierarchy is simplified; the winning crayfish becomes dominant and the losing crayfish becomes subordinate.

Crayfish often fight over resources such as shelter (Ranta & Lindstrom, 1993) or mates (Berril & Arsenault, 1984), however, agonistic bouts persist in the absence of resources (Bruski & Dunham, 1987; Issa et al., 1999). Agonistic encounters between two crayfish begin when one crayfish initiates contact with the other crayfish. Initial contact usually involves striking or grasping the opponent with the first chelae (Bovbjerg, 1953; Bruski & Dunham, 1997; Zulandt et al., 2008). Combatants also perform antennae tapping and whipping (Rubenstein and Hazelett, 1974), which are often combined with urine release (Breithaupt, 2001; Breithaupt & Eger, 2002). A bout is complete when one crayfish breaks off contact with the other, either by retreating from the bout or by performing a tail flip escape (Bovbjerg, 1953; Sato & Toshiki, 2007). The outcome of subsequent fights is determined more quickly. A crayfish that loses one agonistic bout is more likely to lose subsequent bouts with the same opponent and vise versa, a crayfish that wins an agonistic bout is more likely to win subsequent matches (Rubenstein & Hazlett, 1974; Seebacher & Wilson, 2007). In a pairing, after a number of losses the losing crayfish will eventually avoid contact with its opponent to prevent further bouts

(May & Mercier, 2006) and this change marks its status as subordinate (Herberholz et al., 2001).

Factors such as sex, size and prior residence can influence the outcome of crayfish fights. Male crayfish are usually dominant to female crayfish unless female crayfish are maternal (carrying eggs or hatched young), in which case females win significantly more encounters against males (Figler et al., 1995; 2001). Larger opponents, measured using weight, length and chelae size, are more likely to win bouts against smaller opponents (Rubenstein & Hazlett, 1974; Stein, 1976; Ueno & Nagayama, 2012). Crayfish that obtain prior residence in a shelter are also more likely to win encounters against resident intruders (Peeke et al., 1995, Figler et al., 1999). Each of these factors merely influence but do not determine the outcome of encounters. For example, a small crayfish with prior residence can oust a large crayfish while a large crayfish with small chelae may lose an encounter with a small crayfish with large chelae.

After a number of losses, the losing crayfish is deemed subordinate while the winning crayfish is deemed dominant. Dominant crayfish often gain first access to food, shelter and mates (Herberholz et al., 2007). Dominant and subordinate crayfish exhibit changes both physiologically and behaviourally. The muscles involved in tail flip responses have been studied previously. Measuring field potentials in freely moving crayfish, Herberholz et al. (2001) were able to identify four different types of tail flip behaviours. Three types of defensive tail flips are mediated by the lateral giant (LG), medial giant (MG) and nongiant (NG) neurons, respectively. A fourth tail flip, named offensive tail flip (OT) was identified. After 30 min of pairing, dominant crayfish performed more OTs and less NGs than subordinate crayfish. Moreover, the threshold for

a tail flip escape response is increased in subordinate crayfish and decreased in dominant crayfish (Krasne et al., 1997). In fact, when crayfish were injected with 5-HT, EPSPs were inhibited in the lateral giant (LG) neuron of subordinate crayfish and increased in dominant crayfish (Yeh et al., 1997).

Behavioural changes also accompany a change in dominance status. Since a decline in resources often prompts aggressive behaviour in crayfish, the dominant crayfish usually gains access to such resources following success. Dominant crayfish frequently gain first access to food, shelter and mates (Zulandt-Schneider et al., 2001). Dominant crayfish gain access to shelter (May & Mercier, 2006) or to the best shelter if a variety is available (Martin & Moore, 2008). In the absence of a ready-made shelter dominant crayfish perform significantly more burrowing behaviour than subordinate crayfish (Herberholz et al., 2003). This may be an intrinsic drive, rather than an extrinsic bonus to dominance status because Herberholz et al. (2003) found that following the outcome of agonistic interactions, dominant crayfish increased burrowing behaviour while it was suppressed in subordinate crayfish.

Crayfish use visual, chemosensory and tactile information to interact with their environment and this includes agonistic encounters. May and Mercier (2006) chose to isolate visual cues to examine the effect of dominance status in male crayfish. Dominance rank was established by an initial 30 min observation of fighting activity, followed by pairing for two weeks. Dominant and subordinate crayfish were then exposed individually to an aquarium where half of the walls were reflective while the other half were non-reflective. Response to reflection varied depending on dominance status. Dominant crayfish spent more time on the reflective side of the aquarium and spent more

time in reflective corners compared to the non-reflective side. They also performed more cornering, turning and crossing on the reflective side compared to the non-reflective side. Subordinate crayfish performed more reverse walking on the reflective side but showed no other preference for the reflective environment. Further research revealed that this divergence in behaviour was evident after three days of pairing male crayfish (May & Mercier, 2007). The preference for reflection and enhancement of reflection-enhanced behaviours was determined to be the dominant profile of behaviour. The absence of reflection-augmented behaviours was determined to be the subordinate profile of behaviour. It was also revealed (May & Mercier, 2007) that crayfish of unknown dominant status, taken from a community tank and immediately tested in the reflection aquarium exhibited some components of dominant behaviour such as increased time on the reflective side of the tank. Crayfish that were paired for only 30 min exhibited virtually all aspects of dominant behaviour in the mirror/matte tank, regardless of their newly established dominance rank as "winners" or "losers" (May & Mercier, 2007). This suggests that the dominant profile may indeed be the default behaviour. Furthermore, these findings suggest that it is the subordinate crayfish behaviour that changes as a result of the agonistic encounters. I propose that losing crayfish learn to become subordinate and that their response to reflection is dependent upon such learning. I further propose that the agonistic encounters between members of crayfish pairs serve as a training exercise and that the responses to reflection can be used to measure the efficacy of that training.

Research into learning is common in species such as nonhuman primates (Conway & Christiansen, 2001), birds (Brown & Gass, 1993), rodents (Papini & Brewer,

1994; Servatius & Shors, 1994; Commins et al., 2003) and molluscs (Sutton et al., 2002) but is rare with respect to invertebrates and more specifically, crustacea. Weisbord and colleagues (2012) studied associative learning in crayfish *Orconectes rusticus*. They conditioned crayfish to recognize an unfamiliar egg cue by pairing it with food. In a Y-maze, conditioned crayfish took less time locating the arm with the egg cue, compared to crayfish in the control group, thus demonstrating the formation of associative memory. Spatial learning has also been demonstrated in the crayfish *O. rusticus*. Researchers showed that crayfish placed repeatedly into a T-maze learned to escape more rapidly with each placement over the course of five days (Tierney & Lee, 2011). Using tactile cue and food as reinforcement they found that crayfish used place cues and response learning equally. Memory of the maze persisted up to a week following training (Tierney & Andrews, 2013).

Training paradigms that compare one trial learning to multi-trial learning (Bobisud & Potratz, 1976) are commonly used to examine learning, memory, long term memory and long term habituation (Carew et al., 1972; Frost et al., 1985). Multi-trial learning, also known as spaced training, refers to a protocol where training periods are interspersed with periods of rest. One trial learning, also known as massed training, refers to training where one trial is provided, usually of a duration equal to the sum of the multiple trails. The consensus is that spaced training is more successful in inducing learning and memory consolidation than massed training (Melton, 1970; Beck et al., 2000; Menzel et al., 2001). While these training paradigms are commonly used to test learning and memory, they are rarely used to study learning in crustaceans.

Maldonado (2002) examined habituation of the escape reflex in the crab *Chasmagnathus granulates* and found that he could reduce the number of training trials by providing an interval of rest between trials. Crabs that were exposed to trials interspersed with periods of rest exhibited higher levels of retention, during both testing and re-testing, compared to animals that received no inter-trials rest. Using a similar training paradigm, Pedreira et al. (1995) showed that the memory attained by massed training lasted up to two days, while the memory retention lasted up to 5 days in crabs exposed to massed training. Furthermore, cycloheximide abolished the memory acquired after spaced training but not massed training (Hermitte et al., 1999). These findings suggest that massed training produces long-term memory that requires protein synthesis, while spaced training produced intermediate-term memory that does not.

The paper examines the effect of spaced versus massed training on the rate of acquisition of dominance ranks and on responses to reflection, in the crayfish *Procambarus clarkii*. Agonistic encounters are considered to represent a training paradigm and responses to reflection proposed to represent a memory test. This paper tests the hypothesis that acquisition of a subordinate rank is learned. The first prediction from this hypothesis is that losing crayfish will decreased their aggressiveness more rapidly in a spaced trial paradigm than in a massed trial paradigm. The second prediction is that losing crayfish will exhibit a subordinate profile behaviour in response to reflection following a spaced trial paradigm but not following massed training.

2.03: MATERIALS AND METHODS

Eighty adult male crayfish (*Procambarus clarkii*), obtained from Atchafalaya Biological Supply, Co., Raceland, Louisiana, USA, were used in this experiment. All crayfish were intact and no crayfish moulted during this experiment. Crayfish ranged from 18.2g to 37.1g (29.4 ± 8.6; Mean ± SD) and measured, rostrum to telson, 8.1cm to 10.5 cm (9.2 ± 0.5, Mean ± SD) in length.

Crayfish were housed in one of three community tanks, two measuring 145 cm long × 55 cm wide × 49 cm high, and one with a depth of 70 cm and a diameter of 120 cm. Community tanks typically held 30 crayfish and contained large rocks and PVC tubing for shelter. Water in the community tanks was filtered and aerated and was siphoned and replaced once weekly. 40 crayfish pairs were selected based on a similar size and always from different community tanks. Their dominance status was unknown. Crayfish were fed *ad libitum*, three times weekly, with artificial crayfish obtained from local grocers. The wet lab were crayfish were housed was maintained on a 12h light:12h dark photoperiod and both the room and water were 21° C.

Crayfish pairs were placed in separate holding plastic containers that were opaque black, measuring 30cm × 17.5cm × 13cm. One crayfish of each pair was marked with a dot on the dorsal abdominal carapace, using white enamel paint, while the other was marked with a line. Containers were filled with aerated water and each contained a small green flower pot to be used for both shelter and handling of the crayfish. A screened lid, to allow light in, was placed on top. Containers with crayfish were transported to a testing room and held for 30 minutes prior to testing.

2.03.1*: Treatment Groups*

Eighty crayfish were paired and randomly distributed into two groups (20 pairs each). The massed group was paired for 90 consecutive minutes and the resulting fight activity that occurred was filmed. The spaced group was paired for 30 min, separated for 30 min, paired for 30 min, separated again for 30 min and then paired a third timefor 30 min more. This resulted in 90 min of fighting, with two 30 min breaks. The members of each pair were always re-paired together; no crayfish ever met more than one opponent. Aside from the massed vs. spaced training paradigm, crayfish were treated in the same manner in all other aspects of this experiment.

2.03.2: *Dominance Testing*

Crayfish pairs were transferred together, using a flower pot to reduce handling, into a clear plastic container measuring 48cm long × 25cm wide × 13cm high. The container was filled to approximately 9cm with aerated filtered water. Filming began once crayfish were placed in water. Crayfish were filmed from above using a webcam and PC. The crayfish were left alone during filming so that the experimenter did not influence behaviour. Crayfish in the massed group were filmed for 90 min. Crayfish in the spaced group were filmed for 30 min and then each crayfish was placed back into their holding container for 30 min. The crayfish were subsequently placed back into the testing container, filmed for 30 min and moved back to the same holding container for 30 min. Crayfish pairs were then moved back into the testing container and filmed for a final 30 min.

Crayfish fight videos were observed after all testing and video analysis of reflection responses was complete. Behaviours observed and recorded included initiation, approach, striking, grasping, pulling, pushing, retreat, tail flip and avoidance. Each of these behaviours have been reported previously (Bovbjerg, 1953; Copp, 1986; Bruski and Dunham, 1987; Huber and Delago, 1998; Lundberg, 2004) and a full description is reported here (Table 2.1). Typically, agonistic bouts consisted of one crayfish initiating contact with the other, followed by striking, grasping, pushing and pulling. A bout ended when one crayfish would retreat, either by walking away or tail flipping. After many similar fights one crayfish would invariably begin to avoid contact with the other. After 90 min of fighting one crayfish would be deemed dominant, while the other would be deemed subordinate. This was accomplished by calculating a dominance index (Guiasu and Dunham 1997; Goessmann et al. 2000; Bergman et al. 2003) for each pair (Table 2.2), where the proportion of all agonistic encounters won was calculated (DI = # of wins / (# of wins + # of losses)). A crayfish was considered to have won a fight if the opposing crayfish retreated, either by walking away or tail flipping away. The retreating crayfish was determined to be the loser. Mean (±SD) dominance indices in the massed training group were 0.95 ± 0.06 for dominant crayfish and 0.05 ± 0.06 for subordinate crayfish. The means were significantly different (paired t-test, $p < 0.0001$). In the spaced training group, mean (±SD) dominance indices were 0.96 ± 0.07 for dominant crayfish and 0.04 ± 0.07 for subordinate crayfish and the means were significantly different (paired t-test, $p < 0.0001$).

2.03.3: *Testing for Effects of Reflection*

Immediately following crayfish fights, crayfish were tested for their response to a reflective environment. One member of each crayfish pair was placed into a Plexiglass aquarium (52 cm long × 25 cm wide × 30 cm deep) in which half of the perimeter (lengthwise) was covered in mirrors and the other half was covered in a non-reflective clear plastic. The tank was 2/3 filled with aerated filtered water. The crayfish was placed gently in the centre, using the flower pot shelter for handling. Filming from above began immediately and lasted 20 min. Once filming concluded, the crayfish was placed back in the holding container and was returned to the community housing tank. The testing aquarium was rinsed and refilled with aerated filtered water. The second crayfish was then placed into the tank and the procedure was repeated. The crayfish were identified using markings (dot and dash) and the order of testing was counterbalanced for this part of the experiment. The aquarium was rotated between trials to eliminate any preference that crayfish may have had for one side of the room. The researcher was not present during filming, to minimize any external influences.

Several behaviours were observed when analyzing the reflection testing videos. These behaviours are briefly described in Table 2.3. A more detailed description of behaviours has been previously reported by this lab (Drozdz et al., 2005; May & Mercier, 2006; May & Mercier, 2007). Cornering, time spent cornering, turning, crossing, reversing walking and the total time spent on each side of the aquarium are reported here.

Table 2.1. Description of agonistic behaviors.

Behaviour	**Description**
Initiation	One crayfish approaches the other and makes contact.
Approach	One crayfish approaches the other, within one body length, but contact is not made.
Grasping	The crayfish uses its chela(e) to grasp onto the opposing crayfish.
Striking	The crayfish uses its chela(e) to jab or strike the opposing crayfish.
Pushing	The crayfish pushes the opponent at least one body length, using the chelae.
Pulling	The crayfish grasps the opponent with the chelae and pulls him forward at least one body length, sometimes using a tail flip for propulsion.
Flipping	The crayfish grasps the opponent with the chelae and flips it over onto its dorsal carapace.
Retreat	One crayfish retreats from contact, creating at least one body length of space between opponents (excludes tail flip).
Tail Flip	The crayfish performs a rapid tail flip to retreat from contact.

Table 2.2. Dominance Index values for Massed and Spaced crayfish pairs.

Pair	Massed Dominants	Massed Subordinates	Spaced Dominants	Spaced Subordinates
1	0.96	0.04	0.94	0.06
2	0.99	0.01	0.70	0.30
3	0.75	0.25	1.00	0.00
4	0.97	0.03	0.96	0.04
5	0.93	0.07	1.00	0.00
6	0.99	0.01	0.95	0.05
7	1.00	0.00	0.96	0.04
8	0.84	0.16	0.95	0.05
9	0.97	0.03	0.99	0.01
10	0.96	0.04	1.00	0.00
11	0.93	0.07	0.90	0.10
12	0.97	0.03	1.00	0.00
13	0.93	0.07	0.93	0.08
14	0.99	0.01	0.97	0.03
15	1.00	0.00	0.99	0.01
16	0.93	0.07	0.98	0.02
17	0.98	0.03	1.00	0.00
18	1.00	0.00	0.91	0.09
19	0.96	0.04	1.00	0.00
20	0.97	0.03	1.00	0.00

Table 2.3. Description of Behaviours Analyzed

Behaviour	Description
Cornering	Crayfish faces the corner and remains there for a minimum of 5s.
Time Spent Cornering	The total time that the crayfish spends cornering.
Turning	A turn of more than 90° that results in a change of direction, altering the walking path from clockwise to counter clockwise or vice versa.
Crossing	The crayfish leaves the walls of the aquarium and crosses to adjacent or opposing wall. Crosses must take place at least one body length from a corner.
Reverse Walking	The crayfish walks in reverse for a minimum of one crayfish body length.
Total Time	The total time the crayfish spends on the reflective or non-reflective side of the aquarium.

2.04: RESULTS

2.04.1: *Agonistic Behaviours*

Crayfish in the massed and spaced paradigms exhibited similar agonistic interactions. Fights began with one crayfish approaching the other. Bouts typically began slowly, with some strikes, grasps and pushing, and were brief. Bouts escalated in intensity and duration, with crayfish exhibiting a rapid series of strikes and grasps, and occasional pushes and pulls. Tail flip retreats often occurred during agonistic bouts of high intensity. Eventually, one crayfish retreated from the interaction by walking away. After several bouts, one crayfish would approach but not make contact because the opponent would avoid it. Avoidance behaviour was always seen in the losing crayfish and often marked the end of agonistic interactions.

The beginning of each bout was always taken as the time when one crayfish initiated contact with its opponent. Dominant crayfish initiated contact significantly more frequently than their subordinate counterparts in both the massed and spaced groups (Fig 2.1a) (paired t-test, $p<0.001$ for both dominant groups). There was no significant difference between massed dominants and spaced dominants or between massed subordinates and spaced subordinates with respect to fight initiation. Initiations declined over time, indicated by a significant difference in initiations performed in the first 30 min period compared to the last 30 min period (ANOVA, $p<0.0001$; Tukey HSD, $p<0.01$, for all groups). There was also a significant decline in the number of initiations performed in the second 30 min period compared to the last 30 minute period for every group of crayfish (ANOVA, $p<0.0001$; Tukey HSD, $p<0.01$, for all groups).

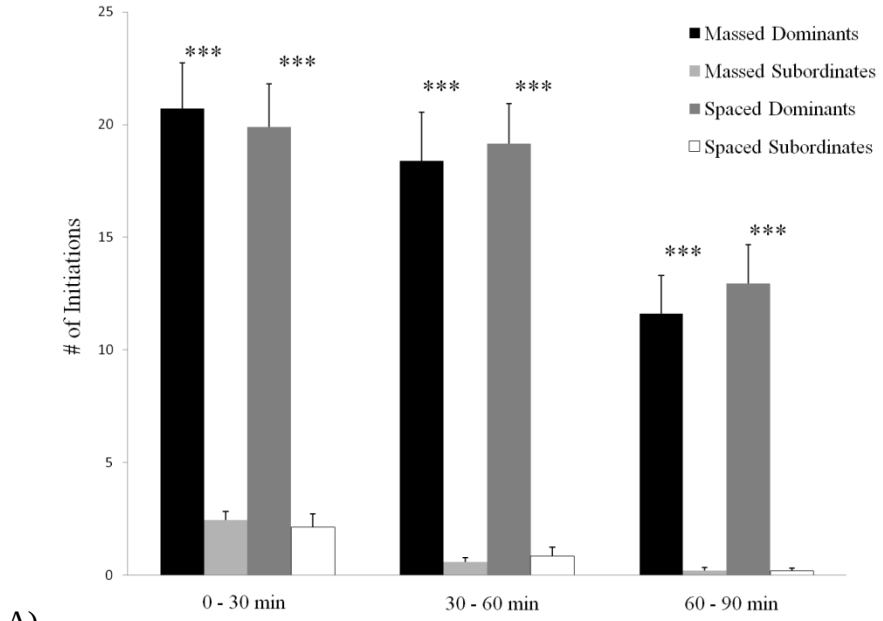
Concurrent with the decline in frequency of initiations, there was an increase in the number of approaches over time. Both massed and spaced dominant crayfish approached their opponent significantly more frequently than did their subordinate counterparts in the first 30 min (Fig 2.1b) (paired t-test, $p<0.001$), as well as the last two 30 min periods (Fig 2.1b) (paired t-test, $p<0.0001$) for every 30 min period. There was no significant difference between the number of approaches performed by massed dominants and spaced dominants, nor between massed subordinates and spaced subordinates, within any time period. Dominant crayfish from the spaced group performed significantly more approaches in the last 30 min period compared to the first 30 min period (ANOVA, $P<0.05$; Tukey HSD, $p<0.05$), while no other crayfish groups exhibited a difference over time in approaches.

During agonistic encounters, dominant crayfish initiated and approached the opponent more frequently than subordinates (Fig 2.1), and alternatively, subordinate crayfish retreated and avoided contact more frequently (Fig 2.2). Subordinate crayfish retreated more frequently than their dominant opponents during every 30 min period (Fig 2.2a) (Paired t-test; $p<0.0001$). Dominant crayfish in the massed and spaced groups performed fewer retreats in the second and third 30 min period compared to the first 30 min period (ANOVA, $P<0.0005$; Tukey HSD, $p<0.01$). Subordinate crayfish in both the massed and spaced groups performed more retreats in the last 30 min period compared to the first two 30 min periods (ANOVA, $P<0.0005$; Tukey HSD, $p<0.01$). While retreats occur when one crayfish ceases contact, avoidances occur when one crayfish avoids contact with the other. Subordinate crayfish avoided contact more frequently than their opponents during the first 30 min period (Paired t-test, $p<0.005$) as well as the last two 30

min periods (Paired t-test, $p<0.0001$) (Fig 2.2b). Dominants in the massed training group performed more avoidances during the first 30 minutes compared to the last two 30 min periods (ANOVA, $P<0.05$; Tukey HSD, $p<0.05$). Subordinates in the spaced training group performed more avoidances during the last 30 min period compared to the first 30 min period (ANOVA, $P<0.05$; Tukey HSD, $p<0.05$)

Striking and grasping are the most frequent agonistic behaviours observed during crayfish fighting and occur during the most intense part of the fight. Within the massed tranining group, dominants performed more strikes than subordinates in every time period (Fig 2.3a) (paired t-test, $p<0.05$, 0 – 30 min and 60-90 min; $p<0.001$, 60-90 min). Within the spaced traning group, dominants also demonstrated more strikes than subordinates for every 30 min period (Fig 2.3a) (paired t-test, $p<0.001$, all 30 min periods). There was no significant difference between the number of strikes across training groups, neither when comparing dominants nor when comparing subordinates. The number of strikes declined over time for every group. All crayfish, regardless of status or group, performed significantly fewer strikes in the second 30 min period compared to the first 30 min period (ANOVA, $P<0.0001$; Tukey HSD, $p<0.01$) and in the last 30 min period compared to the first 30 min period (ANOVA, $P<0.0001$; Tukey HSD, $p<0.01$).

Dominant crayfish exhibited more grasping than subordinate crayfish in the massed training group for every 30 min period (Fig 2.3b) (Paired t-test, $p<0.001$, 0-30 min and 60-90 min; $p<0.001$, 30-60 min). In the spaced traning group, dominant crayfish also performed more grasping than their subordinate opponents in every 30 min period (Fig 2.3b) (Paired t-test, $p<0.05$, 0-30 min; $p<0.001$, 60-90 min, $p<0.0001$, 30-60 min).



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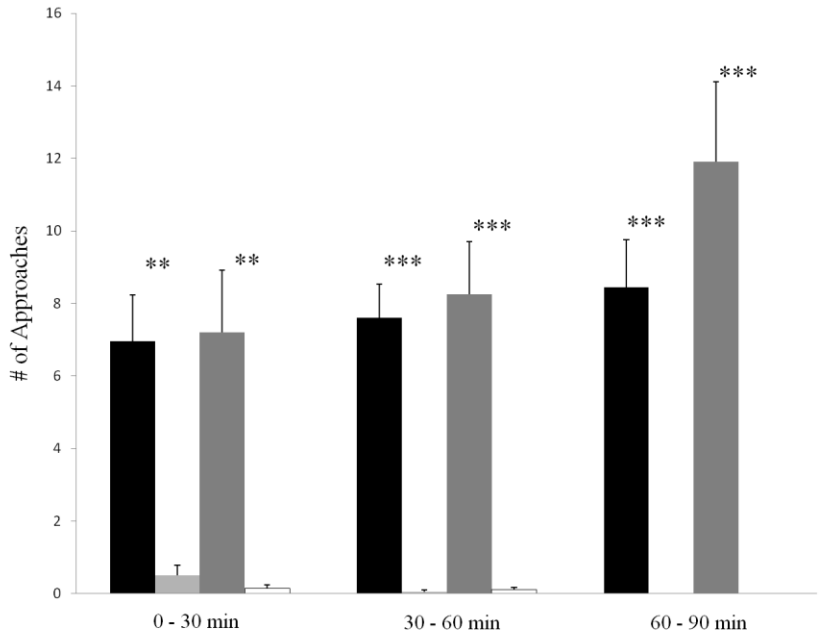
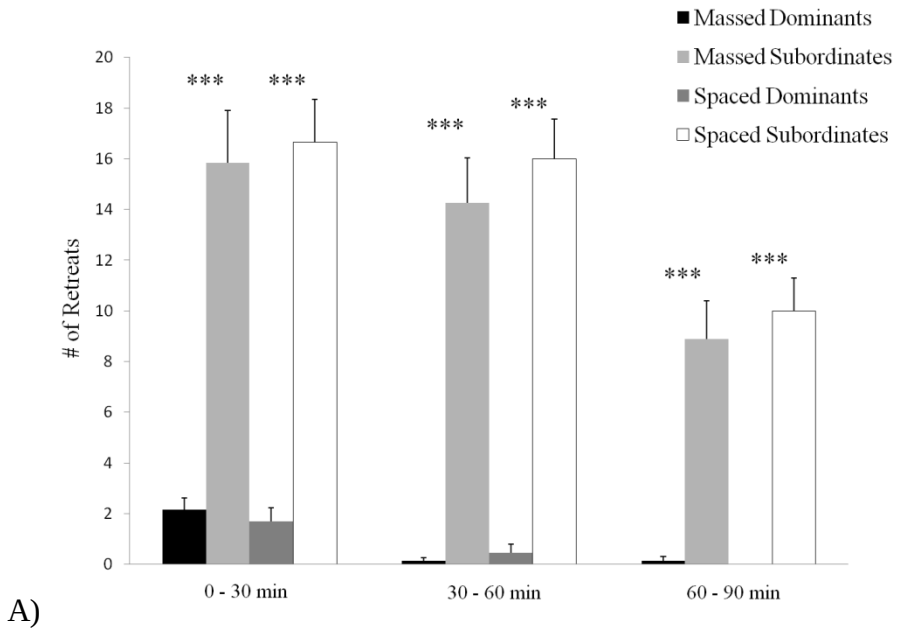


B)

Figure 2.1. The mean number of initiations and approaches during 90 min of observation. Bars depict means ± SEM. (A) Mean frequency of initiations performed by each crayfish group, separated into 30 min periods. Massed Dominant crayfish initiated contact more frequently than Massed Subordinate crayfish and Spaced Dominant crayfish initiated contact more frequently than Spaced Subordinate crayfish (paired t-test, ***$p < 0.0001$). There was no difference between Dominants or Subordinates from either group. (B) The mean number of approaches performed by each crayfish group, separated into 30 min periods. Massed Dominant crayfish approached more frequently than Massed Subordinate crayfish and Spaced Dominant crayfish approached more frequently than Spaced Subordinate crayfish. (paired t-test, **$p < 0.001$, ***$p < 0.0001$).



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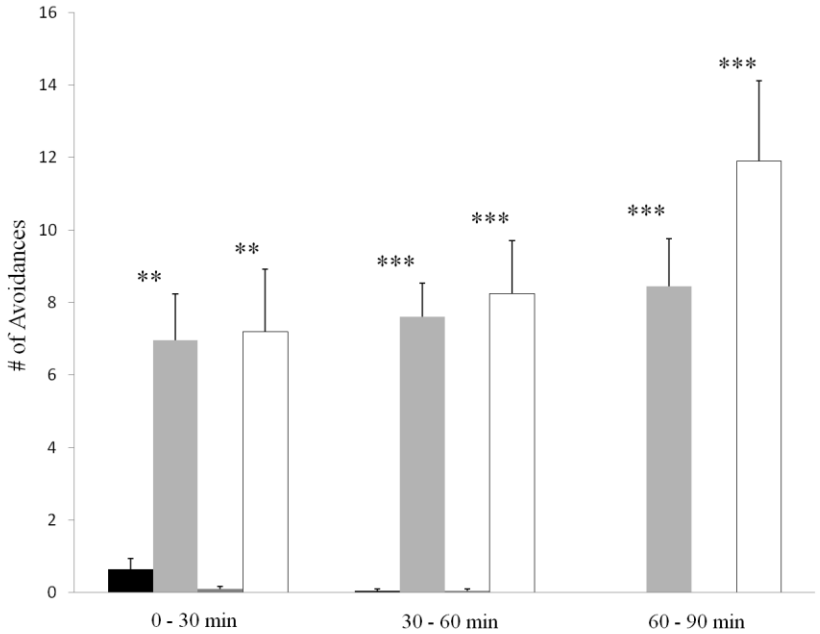
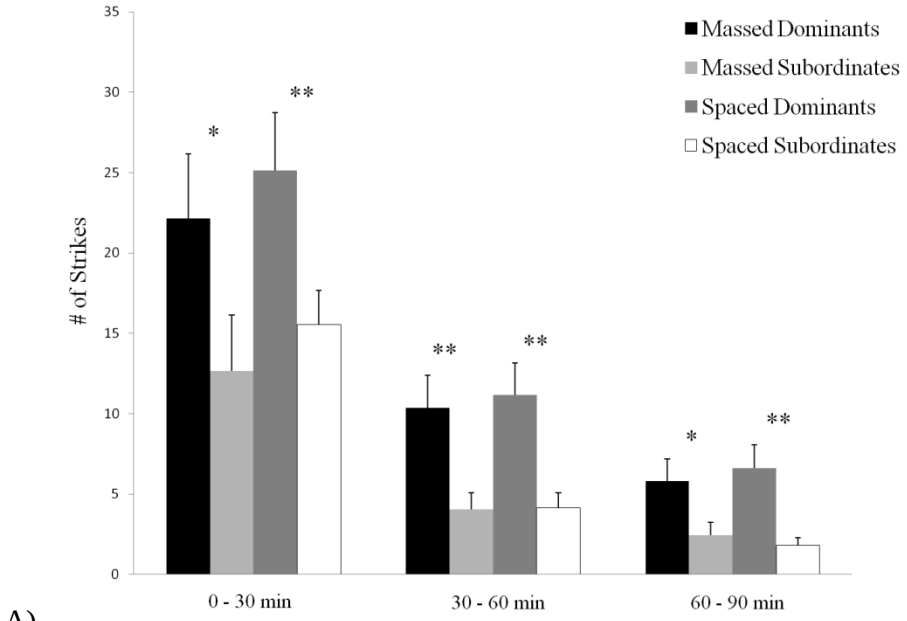


B)

Fig.2.2. The number of retreats and avoidances performed by crayfish during 90 minutes of observation. Bars depict mean ± SEM. A) Both subordinate groups performed more retreats than their dominant opponents during each 30 min period (***$p<0.0001$). B) Both subordinate groups performed significantly more avoidances than dominant crayfish during the first 30 min (**$p<0.005$) as well as the last two 30 min periods (***$p<0.0001$).

There was no significant difference in the number of grasps across training groups neither when comparing dominant crayfish between the massed and spaced groups nor when comparing subordinate crayfish between both groups. As with striking, the number of grasps declined over time. All crayfish, regardless of dominance rank or training group, performed fewer grasps in the second 30 min period when compared to the first 30 min (ANOVA, $P<0.0001$; Tukey HSD, $p<0.01$). They also performed fewer grasps in the last 30 min period compared to the first 30 min (ANOVA, $P<0.0001$; Tukey HSD, $p<0.01$).

To further examine differences in agonistic behaviours, an Aggression Index (AI) was calculated. AI was adapted from a method used by Cromarty et al., (1999) and calculated by taking the sum of aggressive behaviours (initiation, approach, pushing, pulling, flipping, striking and grasping) divided by the sum of aggressive behaviours plus the sum of submissive behaviours (retreat, tail flip and avoidance). AI increased slightly over time for dominant crayfish in both training groups and decreased over time for subordinates in both training groups (Fig 2.4a). There was no significant difference between AI values for dominant crayfish when comparing massed training and spaced training groups. There was also no significant difference in AI values between subordinate crayfish of the two training groups. To further examine the effect of training time on AI, the change in AI for each crayfish was calculated by comparing the last 30 min period to the first 30 min period (Fig 2.4b). When comparing AI values in the first 30 min period to the last 30 min period, only the subordinate crayfish from the spaced group exhibited a significant decline (Mann-Whitney, $P<0.01$).



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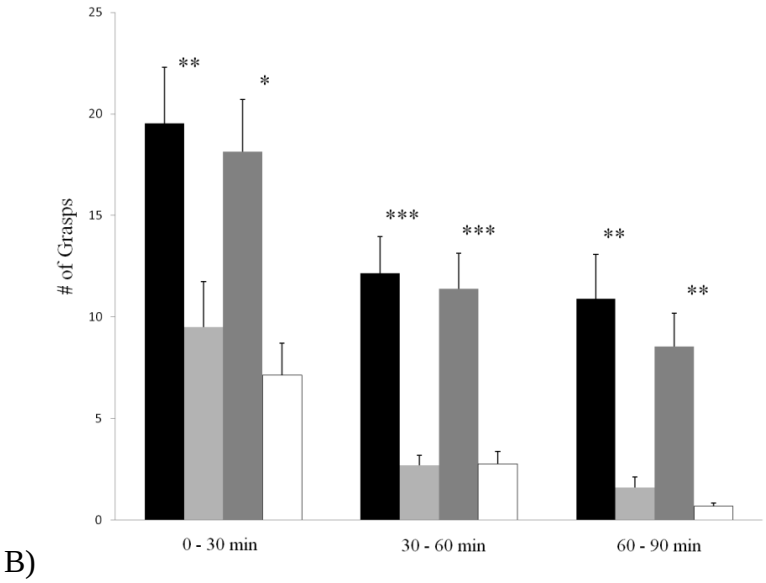
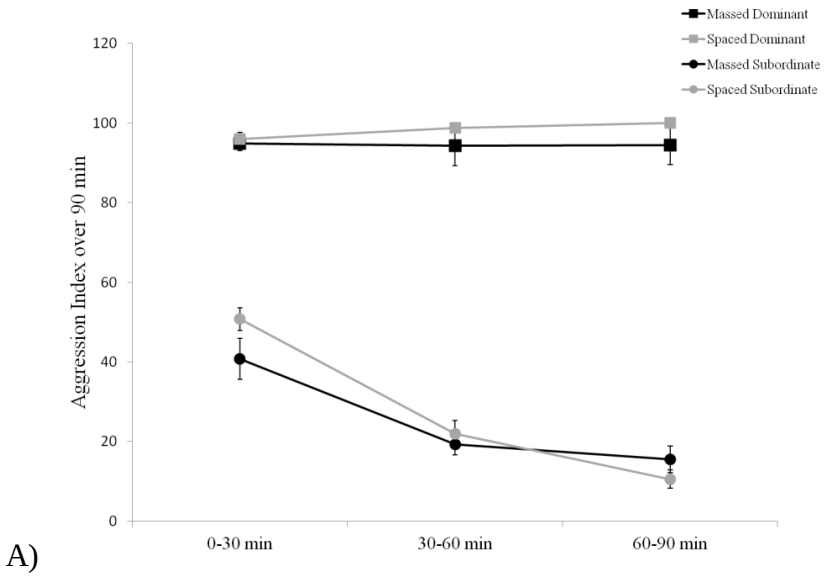
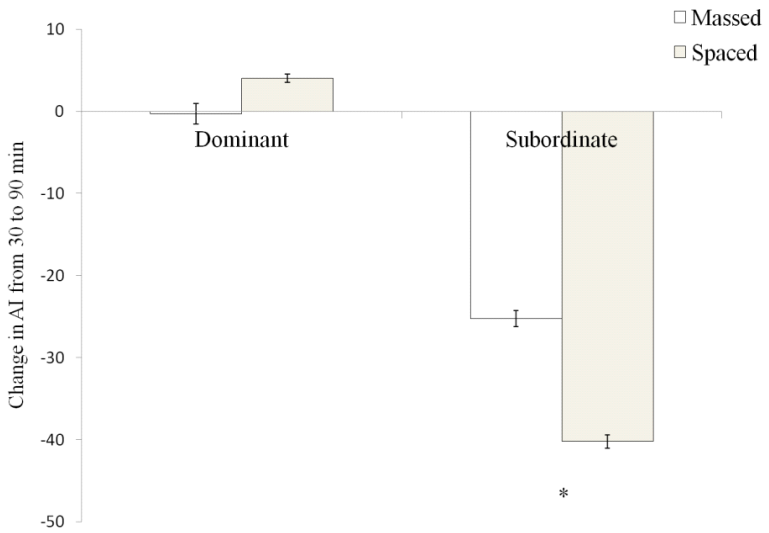


B)

Fig 2.3. The mean number of strikes and grasps performed by each crayfish group during 90 min of observation. Bars depict means ± SEM. A) The mean frequency of strikes performed by crayfish pairs, separated into 30 min periods. Dominant crayfish from both the massed and spaced groups performed more strikes than their subordinate opponents (paired t-test, * $p<0.01$, ** $p<0.001$). B) The mean frequency of grasps performed by each crayfish group, separated into 20 min periods. Dominant crayfish from the massed group performed more grasps than their subordinate opponents and dominant crayfish from the spaced group also performed more strikes than subordinates from the spaced group (paired t-test, * $p<0.01$, ** $p<0.001$, ***$p<0.0001$).



A)



B)

Figure 2.4. Aggression index (AI) calculated for each crayfish group. A) AI declines over time for each crayfish group (error bars depict SEM). There is no significant difference between the massed and spaced groups when comparing AIs. B) Bars depict the change in AI from the first 30 min period compared to the last 30 min period (error bars depict SEM). Only subordinate crayfish from the spaced group showed a significant decline in AI over time (Mann-Whitney, *P<0.01).

2.04.2: *Responses to Reflection*

Crayfish corner more frequently than any other behaviour when in the reflection testing aquarium (May & Mercier, 2006; 2007). Dominant crayfish performed more cornering events on the reflective side of the tank compared to the non-reflective side, regardless of training group (Fig 2.5) (Paired t-test, $p<0.0005$ for both groups). Subordinate crayfish from the massed group also cornered more frequently on the reflective side of the tank compared to the non-reflective side ($p<0.001$) but subordinate crayfish from the spaced group showed no difference in the number of cornering events on the two sides of the test tank (Fig 2.5).

Dominant crayfish from both training groups spent more time cornering on the mirrored side of the tank compared to the matte side (Fig 2.6) (Paired t-test, $p<0.001$ for massed group, $p<0.0001$ for spaced group). Subordinate crayfish from the massed group also spent more time cornering on the mirrored side compared to the matte side (Fig 2.6) ($p<0.01$). Subordinate crayfish from the spaced group, however, did not show any significant differences with respect to time spent cornering.

The frequency of turning was examined for each crayfish group (Fig 2.7). Turning changes the direction of the walking path (Drozdz et al., 2006; May & Mercier, 2006) and occurred most often at the corners or the mid-point of the aquarium, where the environment changed from reflective to non-reflective or vise versa. Dominant crayfish from both the massed group (Paired t-test, $p<0.05$) and the spaced group ($p<0.001$) performed more turns on the reflective side. Although trends in the data suggested that subordinate crayfish from the massed group turned more frequently on the mirrored side

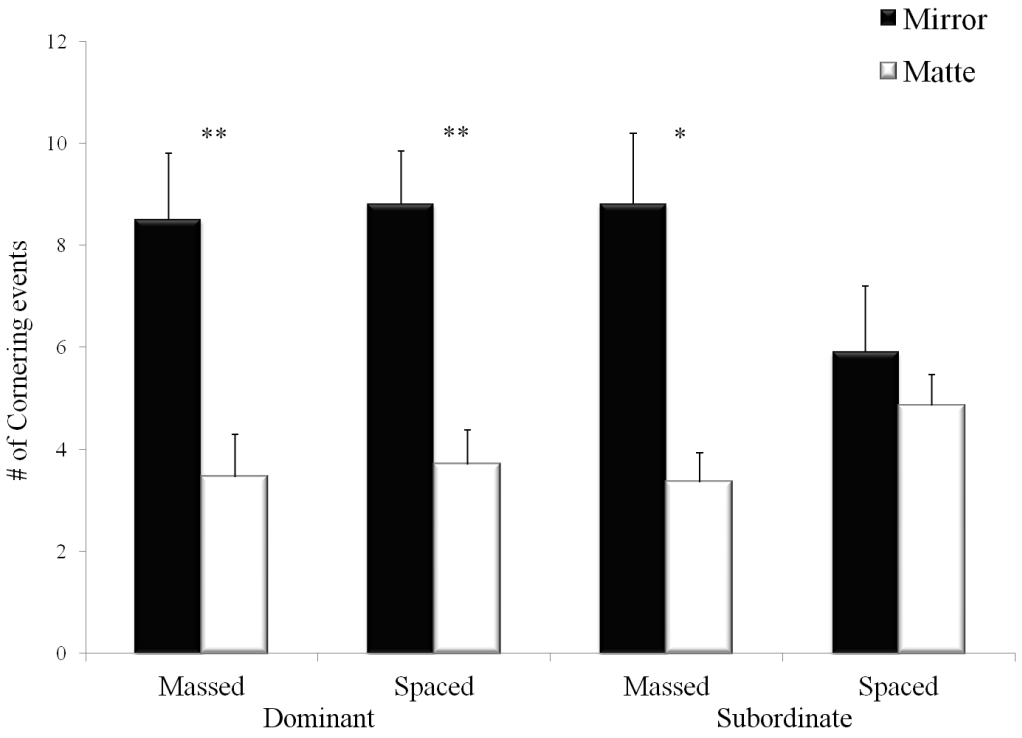


Fig 2.5. The frequency of cornering exhibited by all crayfish groups in the reflective side of the tank (mirror) compared to the non-reflective side (matte) during 20 min of observation. Bars depict the mean ± SEM. Dominant crayfish from the massed and spaced groups cornered more frequently on the reflective side of the aquarium compared to the non-reflective side (**$p<0.0005$). Subordinate crayfish from the massed group cornered more on the reflective side of the tank (*$p<0.001$) but subordinate crayfish from the spaced group showed no significant differences.

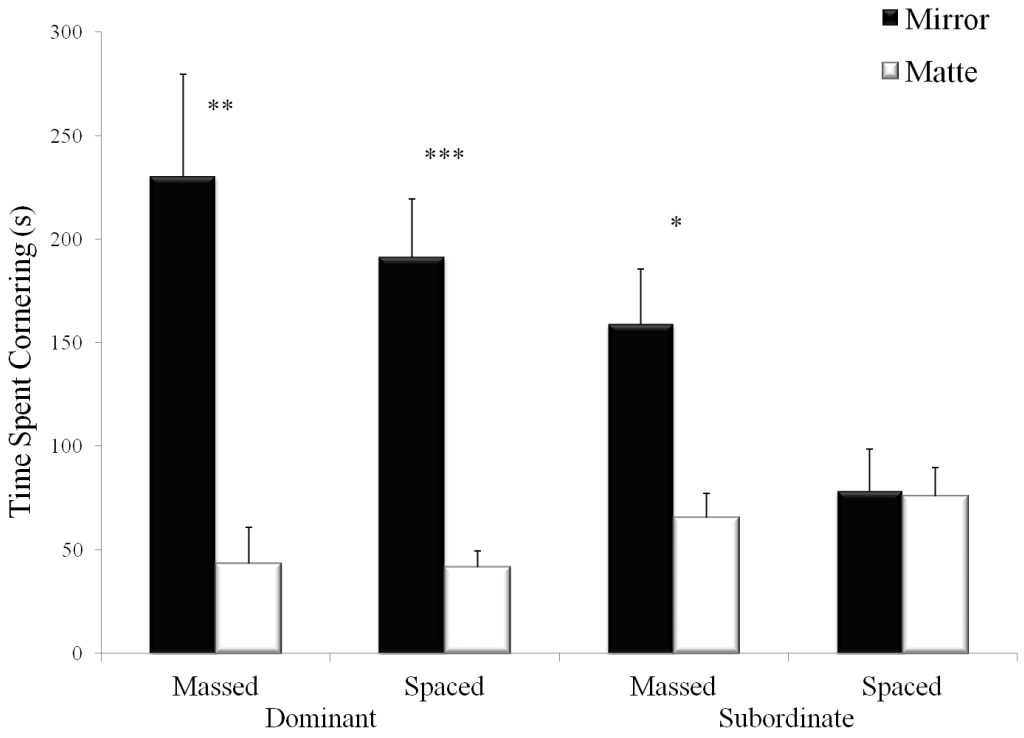


Fig 2.6. Time spent cornering on the mirrored side of the testing chamber compared to the matte side during 20 min of observation. Bars depict mean ± SEM. Massed dominant crayfish (**$p<0.001$) and Spaced dominant crayfish (***$p<0.0001$) spent more time cornering on the mirrored side of the tank versus the matte side. Subordinate crayfish from the massed group also spent more time cornering on the mirrored side of the tank (*$p<0.01$). Subordinate crayfish from the spaced group showed no significant differences.

and subordinate crayfish from the spaced group turned more frequently on the non-reflective side of the tank, neither group showed a significant difference in turning between the two sides of the tank.

Crayfish occasionally performed reverse walking during the observation period. Subordinate crayfish from the spaced group performed more reverse walking events on the reflective side of the test aquarium compared to the non-reflective side, but subordinates from the massed group showed no significant difference in reverse walking between reflective and non-reflective sides (Fig 2.8) (Paired t-test, $p<0.05$). Dominant crayfish from the spaced and massed groups failed to show any significant differences in reverse walking between the two sides of the test aquarium.

The amount of time that each crayfish group spent on the reflective versus the non-reflective side of the test tank was also measured (Fig 2.9). Both dominant and subordinate crayfish from the massed group spent more time on the reflective side of the aquarium compared to the non-reflective side (Paired t-test, $p<0.01$). The dominant crayfish from the spaced group also spent more time on the reflective side of the tank compared to the non-reflective side ($p<0.0001$) but the subordinate crayfish showed no significant differences.

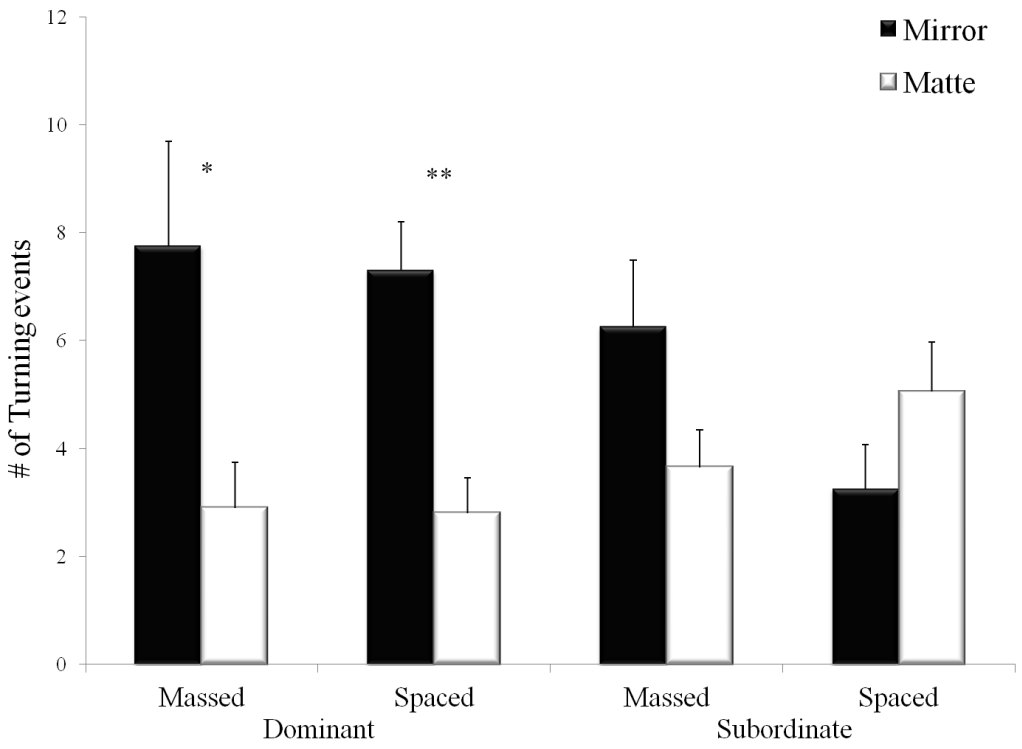


Fig 2.7. The frequency of turning events exhibited by all crayfish groups during 20 min of observation.. Bars depict mean ± SEM. Dominant crayfish from the massed group turned more frequently on the mirrored side of the tank compared to the matte side (*$p<0.05$). Dominant crayfish from the spaced group also turned more on the mirrored side of the tank compared to the matte side (**$p<0.001$). Subordinate crayfish from neither group showed any significant differences.

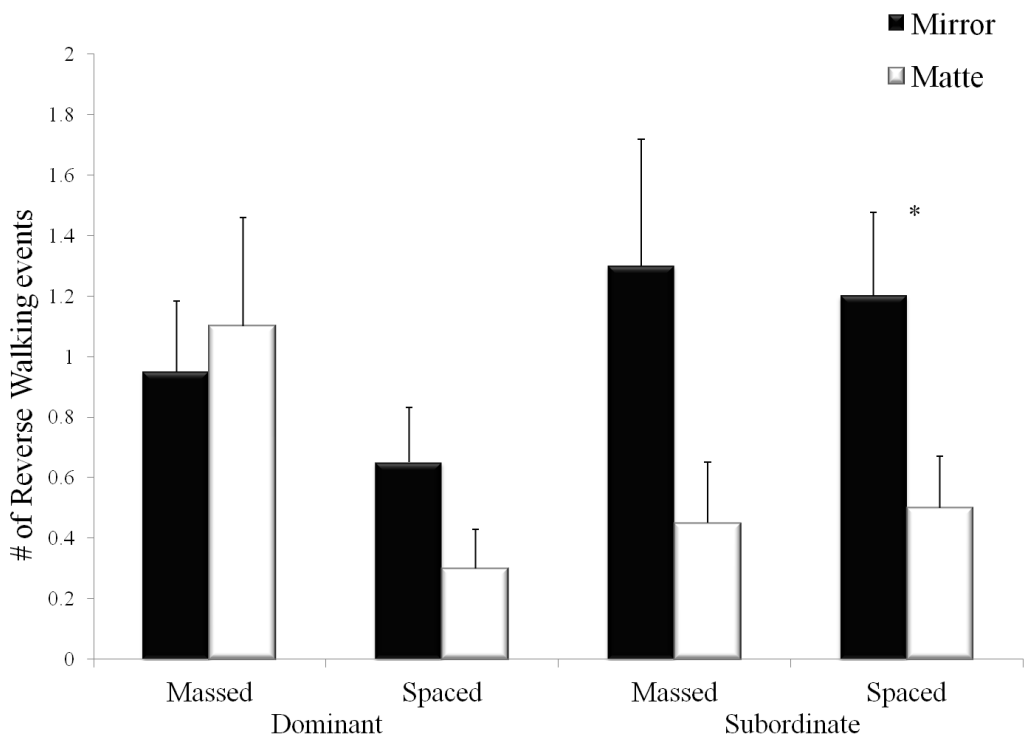


Fig 2.8. The mean number of reverse walking events performed by each crayfish group during 20 min of observation. Bars depict mean ± SEM. Subordinate crayfish from the spaced group exhibited more reverse walking on the non-reflective side of the tank compared to the mirrored side (*$p<0.05$). No significant differences were exhibited with any other group of crayfish.

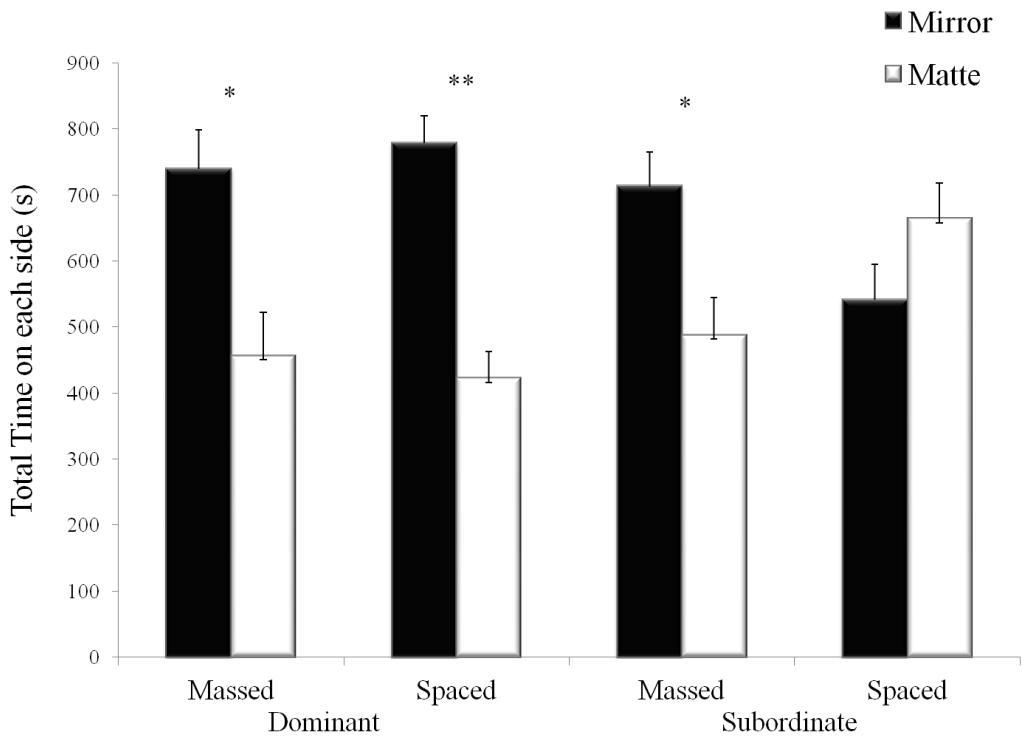


Fig 2.9. The amount of time each crayfish group spent on either side of the aquarium during 20 min of observation. Bars depict mean ± SEM. Dominant crayfish from the massed group (*$p<0.01$) and the spaced group (**$p<0.0001$) both spent more time on the mirrored side of the tank compared to the matte side. Subordinate crayfish from the massed group also spent more time on the mirrored side of the tank (*$p<0.01$), while subordinate crayfish from the spaced group showed no differences.

2.05: DISCUSSION

Previous research has shown that dominant crayfish paired for three days or more, as well as dominant and subordinate crayfish paired for three days or less exhibit a dominant profile of behaviour in response to reflection (May & Mercier, 2006; 2007). That is, they corner, turn and cross more frequently in a reflective environment compared to a non-reflective environment. They also spend more time cornering and more time overall in a reflective environment compared to a non-reflective environment, when given the choice between the two. Subordinate crayfish paired for three days, however, showed no preference for reflection and no enhancement of reflection-dependent behaviours (May & Mercier, 2006), and subordinate crayfish paired for two weeks performed more reverse walking in a reflective environment compared to a non-reflective environment (May & Mercier, 2007). These findings suggested that crayfish respond to their reflected image as a conspecific, with dominant crayfish consistently approachining and interacting with the reflected image and subordinate crayfish learning to avoid the mirror image as they would an opponent.

The mirror image provides visual feedback but not chemosensory and tactile feedback that the crayfish would receive were it engaging a conspecific. This may be why responses to reflection mimic some behaviour seen during actual crayfish encounters, behaviours such as tail flip escape are not observed. The present report proposes that losing crayfish learn to become subordinate and that their response to reflection is dependent upon that learning. The hypothesis led to the predictions that subordinate crayfish would change their responses to reflection, from a dominant to a subordinate behavioural profile, more rapidly following spaced training compared to massed training.

The results support this hypothesis. Subordinate crayfish in the massed training group exhibit dominant responses to reflection, including more time spent on the reflective side as well as more cornering events and longer cornering times on the reflective side, but subordinate crayfish in the spaced training group did not exhibit these dominant behaviours. Interestingly, subordinate crayfish that were exposed to spaced training performed more reverse walking in the reflective environment compared to the non-reflective environment. This was previously observed in crayfish that were paired for 14 days but not crayfish paired for three days (May & Mercier, 2006; 2007). This finding suggests that reverse walking in a reflective environment may be indicative of the robustness of the subordinate status.

In this study the duration of agonistic encounters remained the same for both massed and spaced groups. Spaced groups were provided a 30 minute break in-between three 30 min trials, while massed groups were exposed to one 90 min trial. The fights were examined to determine if there was a difference in the fight dynamics, duration or intensity of fights between the two groups. The data revealed no differences in the number of strikes, grasps, approaches, initiations, retreats or avoidances exist, overall or during individual 30 min periods, between the two treatment groups. The total number of behaviours performed and the ranked intensity of fights were also analysed and revealed no significant differences between groups (Appendix I). An aggression index (AI), using the number of aggressive and submissive behaviours, was calculated and no difference between groups, for any of the 30 min training intervals, was found. There was also no decline in the AI over time, for either group. A change in AI, from the first 30 min period to the last 30 min training period, did reveal a difference between groups. The AI of

subordinate crayfish exposed to spaced training was significantly reduced from the first 30 min period to the last 30 min period. Dominant crayfish from both the massed and spaced groups, as well as subordinate crayfish from the massed group showed no change in AI. While this finding suggests that subordinate crayfish are less aggressive after 90 min of spaced training compared to 90 min of massed training, the data reveal nothing that indicate why this change has taken place. Further experiments, using more training trials of shorter duration may help to elucidate the reason for these changes.

It has been demonstrated in crab that spaced training allows time for memory consolidation and evokes long term memory, while massed training evokes intermediate-term memory (Pedreira et al., 1995; Hermitte et al., 1999). While the persistence of the effect produced by spaced training reported here was not measured, it is presumed that the intervals between training periods allowed time for memory consolidation. The divergence between dominant and subordinate crayfish response to reflection was previously demonstrated after a minimum of three days of pairing (May & Mercier, 2007). While crayfish were videotaped during the first 30 minutes of pairing, no observations took place during the subsequent three days of pairing. Since we know that crayfish fighting declines over time and drops to low levels after 24 hours (Issa et al., 1999) and that crayfish exhibit periods of rest that resemble sleep (Ramon et al., 2004) we can safely assume that crayfish experienced periods of rest during the three days of pairing to allow for memory consolidation. Prior to this study, pairing of less than three days has not been examined, therefore, the time required for memory consolidation following a single trial of fighting is not known.

While the experiments presented here have illustrated that spaced fighting intervals were successful in establishing subordinate crayfish within a short period of time, further experiments are required to fully understand the effect of spaced training on learning in crayfish. Since responses to reflection only mimic conspecific encounters, experiments that examine the effect of spaced training on agonistic encounters with conspecific crayfish are recommended.

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CHAPTER 3:

RESPONSES TO REFLECTION DEPEND ON SOCIALIZATION IN *DROSOPHILA MELANOGASTER*

3.01: ABSTRACT

The present study demonstrates, for the first time, that the fruit fly, *Drosophila melanogaster*, is attracted to mirrors. Given the choice, fruit flies spent significantly more time oriented toward a reflective surface than a non-reflective surface. The present work also characterized the behaviours performed by adult male flies in a test chamber consisting of two mirrored walls and two glass walls to determine whether some behaviours are influenced by reflection. Adult males exhibited behaviours that resembled grooming and courtship activity in the test chamber; reflection had no effect on the frequency of most of these behaviours. Adult male flies that had been reared in isolation behaved differently than did males that were reared in a social environment, but these differences were not related to reflection. The results extend our knowledge about responsiveness of invertebrate animals to reflection, and the implications of the findings for *Drosophila* behaviour are discussed.

3.02: INTRODUCTION

Responses of animals to their mirror image have been examined for decades. When placed in front of a mirror, animals of various species typically exhibit behaviours that can be classified into one of three response types: a) recognition of the mirror image as "self", b) recognition of the mirror image as a conspecific or c) failure to recognize or respond to the mirror image.

Self recognition was first demonstrated in chimpanzees (Gallup, 1970). Initially, when presented with a mirror, chimpanzees responded with social behaviours such as vocalizing and threatening. Social responses decreased over time, while self-directed behaviours, such as grooming, increased over time. To show that chimpanzees do, in fact, demonstrate self-recognition in the presence of a mirror image, Gallup designed the Mark Test. A mark made from red dye was applied above the eyebrow and on the ear of each chimpanzee, durng anaesthesia. Gallup found that chimpanzees performed significantly more mark-directed behaviours in the presence of a mirror than before the mirror was presented. Interestingly, mark-directed behaviour in macaque monkeys was non-existent and Gallup concluded that chimpanzees have an advanced form of intelligence that includes self-concept, which was previously only reported in humans (Gallup, 1970).

Since Gallup's initial experiment, the Mark Test has been used to demonstrate self recognition in other great apes, such as orangutan, bonobo and gorilla (Inoue-Nakamura, 1997), and in bottlenose dolphin (Marten & Psarakos, 1995; Reiss & Marino, 2001), killer whales (Delfour & Marten, 2001), elephants (Plotnik et al., 2006) and most recently in magpie (Prior et al., 2008). Each of these animals exhibited self-recognition as

demonstrated by a variation of the Mark Test. Hundreds of other vertebrate animals have been studied and have failed the Mark Test, which has lead to criticism of the test (de Waal, 2008). It has been suggested that while some animals may recognize self in the mirror, they may not be interested in the mark. Other animals rely more heavily on their other senses, such as smell or taction and a visual test of self-recognition may not be appropriate. To date, no researchers have been able to show self-recognition in an invertebrate model system.

Few invertebrates have been used to study responses to a mirror image, and those that have, appear to respond to their mirror image as they would to a conspecific. Female cuttlefish, *Sepia officinalis*, responds to its mirror image by displaying a "splotch" body pattern, a behaviour only previously reported as a response to female conspecifics (Palmer et al., 2006). This is evidence that female cuttlefish can recognize sex, which is indistinguishable visually for this species by humans, using only visual cues. The response of the hermit crab (*Pagurus marshi*) to a mirror image depends on the amount of detritus covering its shell. Crabs that had the detritus and biota removed from their shell responded to their mirror image with social displays typically seen when responding to a conspecific, whereas crabs whose shells were camouflaged did not display social behaviours (Dunham et al., 1986). Lastly, responses to reflection in crayfish, *Procambarus clarkii*, depend on dominance status (May & Mercier, 2006; 2007). Both dominant and subordinate crayfish appear to respond to a mirror image as they would to a conspecific. That is, dominant crayfish are attracted to reflection, spending more time cornering, turning and crossing in a reflective environment than in a non-reflective environment. Subordinate crayfish reverse walk ("backing away") more frequently in a

reflective environment compared to a non-reflective environment, which may be indicative of a retreat. This is the extent of available research examining the responses of invertebrate animals to their mirror image. The present study is the first to examine the responses of an insect species to reflection. *Drosophila melanogaster* was selected as the species to study because of its frequent use as a model organism for investigations in genetics, behaviour and neuroscience.

Fruit flies attend to visual cues while flying (Tang & Guo, 2001; Budick et al., 2007) and can use visual cues to learn (Ofstad et al., 2011). Fruit flies exhibit social behaviours, including very stereotyped courtship (Markow & Hanson, 1981; Dankert et al., 2009) and aggressive behaviours (Dow & Von Schilcher, 1975; Nilsen et al., 2004). Using a variety of experimental chambers containing mirrors, this investigation sought to determine whether or not *D. melanogaster* is attracted to reflection, as reported for other invertebrates. The experiments were performed with adults flies, and comparisons were made between wild type (WT) Canton S flies and NorpA P24 flies, which are blind. A description and quantification of behaviours performed in response to reflection are reported here.

3.03: MATERIALS AND METHODS

3.03.1: *Fly Stocks and Fly Isolation*

Wild-type Canton S and NorpA P24 *Drosophila melanogaster* were obtained from Bloomington Stock Center (Bloomington, IN). Stock vials were maintained on Jazz-Mix *Drosophila* medium (Fisher Scientific Co.). Flies were housed in an incubator, and kept at 21°C on a 12:12 light:dark cycle. Fruit flies that were taken from a stock vial containing both male and female flies are referred to here as socialized flies. Socialized flies were aspirated into a 1.5mL Eppendorf tube, enclosed with cotton batten moistened with distilled water. Fruit flies were sexed by viewing under a dissecting microscope. Isolated flies were sexed as ferrate pupae and each was transferred to an individual 1.5mL Eppendorf tube, enclosed with cotton batten moistened with distilled water. Cotton was kept moist for the duration of housing. All experiments were performed 1-3 days post eclosure and no flies were tested more than once.

3.03.2 *Electrophysiology*

Electroretinograms (ERGs) were recorded from both wild-type Canton-S and NorpA P24 mutant fruit flies. Recordings were obtained using a glass micropipette pulled on a Kopf Model 700D vertical electrode puller (David Kopf Instruments, Tujunga, CA). The pipette, filled with 1M NaCl, was inserted just below the surface of the eye, and a reference electrode (AgCl-coated silver wire) was placed into the dorsal abdominal wall. Fruit flies were dark-adapted for 5 minutes prior to recording. ERGs were initiated by shining a light from a light source (Model I-150, CUDA Products Corp.) through a 3.5 mm fiber optic cable directed at the fly. Electrical potentials were detected using a Model

IE-201 Intracellular Electrometer (Warner Instruments Corp., Hamden, CT). Signals were digitized and acquired on a PC-compatible computer using a data acquisition system and custom-designed software (Technical Services Division, Brock University) using a sampling rate of 20 kHz.

3.03.3: *Attraction to Reflection Experiments*

3.03.3.1: *Petri Dish Arena*

160 fruit flies (40 male Canon-S, 40 female Canton-S, 40 male NorpA, 40 female NorpA) were sexed and transferred to individual Eppendorf tubes prior to experimentation. Male and female flies were used in this experiment to see if they differed in their response to reflection. The cotton lid was removed, each fly was gently tapped into the experiment arena and the arena lid was closed to secure the fly inside.

The observation arena consisted of a 60mm × 15mm petri dish (Sigma-Aldrich) (Fig 3.1C). The dish was filled with 10 mm of clear silicone elastomer (Sylgard, Dow Corning), to allow a 5mm clearance for the fly. The small clearance allowed flies to flip from top to bottom and vice versa but discouraged exploration of the sides. In half of the experiments (20 flies from each group), the arena was placed on either a mirror or a white sheet of paper, and flies were filmed for 10 min from above with a camcorder (Sony). In the other half of the experiments (20 flies from each group), a mirror or white sheet of paper was placed on the top of Petri dish and flies were filmed from below with the camcorder. This counter-balancing was done to remove any bias due to the fruit fly's possible preference for orientation to gravity. The chamber was swabbed with ethanol and allowed to dry between trails. Videos were recorded and viewed on a PC using

Windows Media Player. Videos were observed and the amount of time the fly was in the up or down position was calculated.

3.03.3.2: *Cubed Arena*

This observation arena consisted of a cube (37.5 mm × 25 mm × 1 mm) placed within a petri dish (10 cm diameter) (Fig 3.1B; modified from Chen et al., 2002). Two walls were constructed with cut microscope slides (1mm thick) and were painted with white acrylic paint on the outside. The other two walls were constructed with mirrors (3mm thick). The arena was placed on white filter paper (6cm diameter), moistened with distilled water, centered in the bottom half of a petri dish (10cm) . The lid of the Petri dish was placed on top of the arena and contained small holes for ventilation and a larger insertion hole.

20 socialized adult males and 20 isolated adult males were aspirated into the arena individually through the insertion hole, which was then covered with a small piece of wax. The arena was filmed for 20 min from above, using a camcorder (Sony). The videos were saved and observed on a PC computer using Windows Media Player. The amount of time that each fly spent on the white walls versus the mirrored walls was calculated from the video recordings.

3.03.4*: Responses to Reflection Experiments*

A third type of observation arena was constructed to allow for close-up videotaping of fruit fly behaviour. The chamber is as described above, with the exception of the walls. Two walls were constructed using mirrors while the other two walls were constructed using 1mm thick clear microscope slide glass (cut to 37.5 mm × 25 mm)(Fig 3.1a). The arena was placed on a Petri dish (10 mm diameter) filled with agarose gel, to provide humidity. Socialized adult males (n=20) and isolated adult males (n=20) were aspirated into the experimentation arena individually and filmed. A fluorescent light was placed 30 cm above the area, which helped to minimize reflection in the glass walls. Between trials, the arena was swabbed with ethanol and allowed to dry, and the agarose gel was replaced. Video recordings were made with a camcorder (Sony) placed in front of the glass walls; each recording lasted 20 min. This arena design and camera placement allowed the observer to view all four walls simultaneously. Videos were saved and viewed on a PC using Windows Media Player. Any behaviour observed was recorded and described. The total number of all behaviours observed was recorded as was the amount of time the fruit fly spent on the clear walls versus mirrored walls. A full description of behaviours is provided in Table 3.1.

Lighting for all experiments was provided by fluorescent ceiling lights and a single fluorescent tube light. The single tube light was placed directly above the experimental set-up to reduce reflection on non-reflective walls. The lighting remained stable for all experimental conditions.

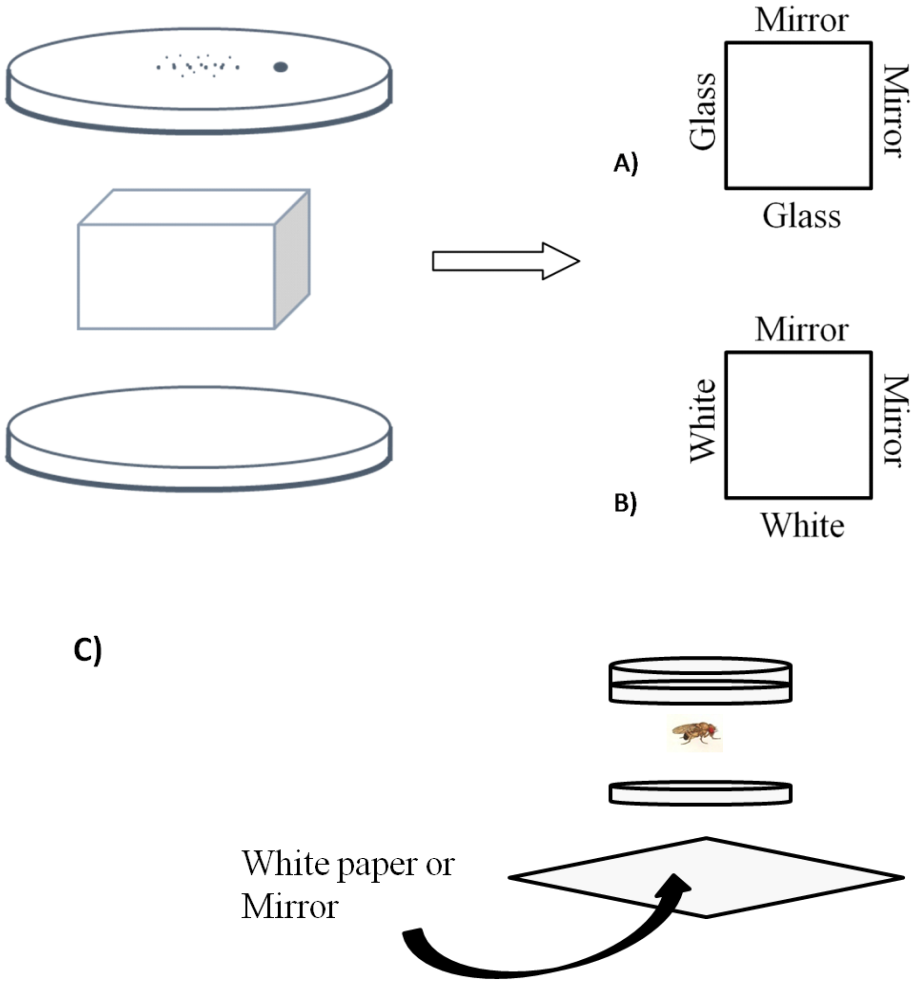


Fig 3.1. Test arena used for fruit fly observation. Walls measure 37.5 mm × 25 mm × 1 mm. Petrie dish has a diameter of 25 cm. A) This arena has two walls constructed with mirrors and two constructed from clear microscope slide glass. The camera was placed in front of the glass walls, so that the glass corner was centered in the film. B) This arena was constructed with two mirrors and two pieces of glass that were painted white acrylic paint on the exterior surface. The camcorder was placed above the arena for filming. C) This arena consists of a 50mm Petri dish filled with Sylgard to 5mm clearance with the lid on. The arena was placed on either a white sheet of paper or a mirror and filmed from above.

Table 3.1. Description of behaviours performed in Reflection Response Arena.

Behaviour	**Description**
Proboscis Extension	The fly extends its proboscis. This is a taste behaviour (Shiraiwa & Carlson, 2007) When proboscis extension is associated with courtship it is often referred to as "licking" (Yamamoto & Koganezawa, 2013).
Abdominal Tapping	The fly bends the abdomen, tapping the end on the substrate. This behaviour has not been previously described in literature but resembles attempted copulation (Sokolowski, 2001).
Front Leg Grooming	The fly balances on its rear legs while rubbing the fore legs together (Kain et al., 2012).
Rear Leg Grooming	The fly balances on its front legs while rubbing the hind legs together (Kain et al., 2012).
Wing Scissoring	The fly extends both wings and moves them across one another in a lateral direction (scissoring motion) (Ewing & Bennet-Clark, 1968; Hegde & Krishna, 1997).
Wing Vibration	A single wing is extended laterally and vibrated in a ventral-dorsal direction, relative to the abdomen (Ewing & Bennet-Clark, 1968).

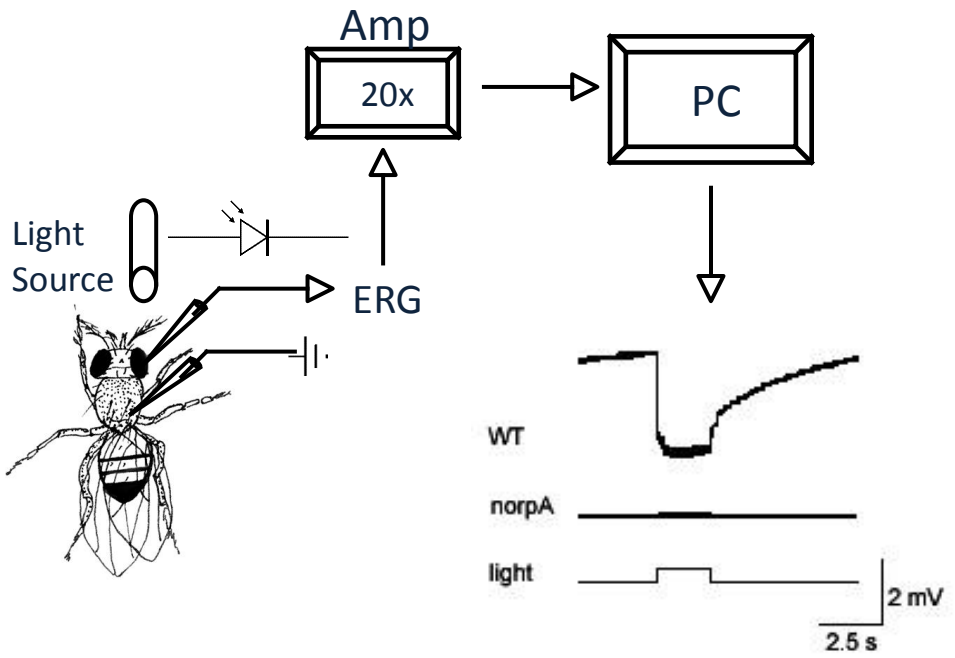
3.04: RESULTS

3.04.1: *Electroretinograms*

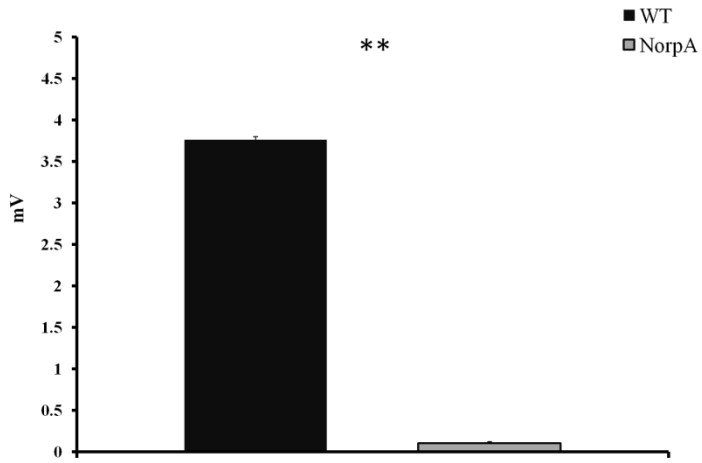
NorpA P24 mutants were used in these experiments as a control for visual cues. NorpA P24 fruit flies have a mutation in the enzyme phosopholipase C (PLC) expressed in the retina, rendering the fly blind due to a build up of rhodopsin and an inability to convert it to meta-rhodopsin (Kim et al., 2003). An electroretinogram was completed to ensure that the flies lacked phototransduction. Fig. 3.02 provides a schematic diagram of the experimental set-up, examples of electrical activity recorded and a comparison of ERG activity between wildtype Canton S and NorpA P24 mutants. The experiments confirmed the inability of the mutant fly to respond to light.

3.04.2: *Reflection Attraction Experiments*

In the first series of trials, male and female fruit flies were filmed individually in the Petri dish arena. Each fly was exposed to either a white sheet of paper or a mirror placed under or over the arena. Male WT fruit flies spent significantly more time oriented towards the mirror than the away from the mirror during 10 min of observation (Fig. 3.3) (Paired t-test, $p<0.0001$). WT males did not show a preference for paper and NorpA males did not show a preference for either paper or the mirror. Female WT fruit flies also spent significantly more time oriented towards the mirror versus away from the mirror (Fig. 3.4) (Paired t-test, $p<0.0001$). Female NorpA fruit flies did not show a preference for the mirror and neither WT nor NorpA females showed a preference for the white paper.



A)



B)

Fig. 3.2. Electroretinogram (ERG) of wildtype and NorpA fruit flies. A) A representation of the experimental setup with an example of ERG recordings from wildtype Canton S male flies (WT) and NorpA P24 male flies (NorpA). B) Graph depicting results from ERG and the inability of the NorpA eye to respond to light. (WT, n=12; NorpA, n=6). The eye of wildtype flies responds significantly more to light than NorpA flies (**P<0.001).

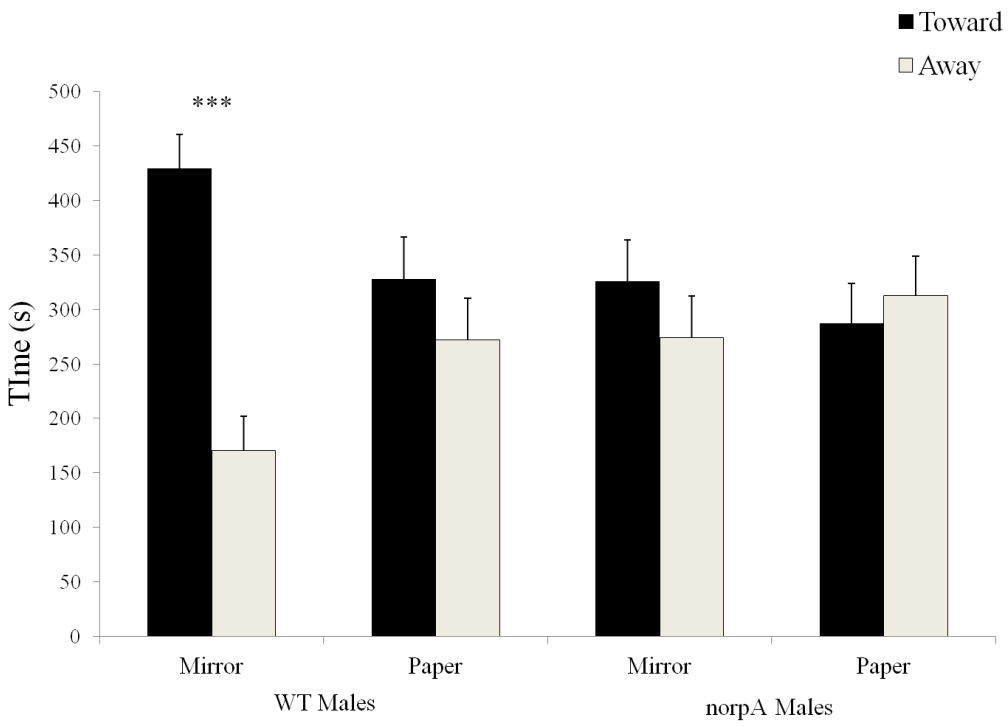


Fig. 3.3: The total time (s) that male fruit flies spent oriented towards or away from the stimulus during 10 min observation in the Petri dish arena. Bars depict mean ± SEM; n=40. Wild-type (WT) flies spent significantly more time oriented towards the mirror compared to away from the mirror (***p <0.0001).

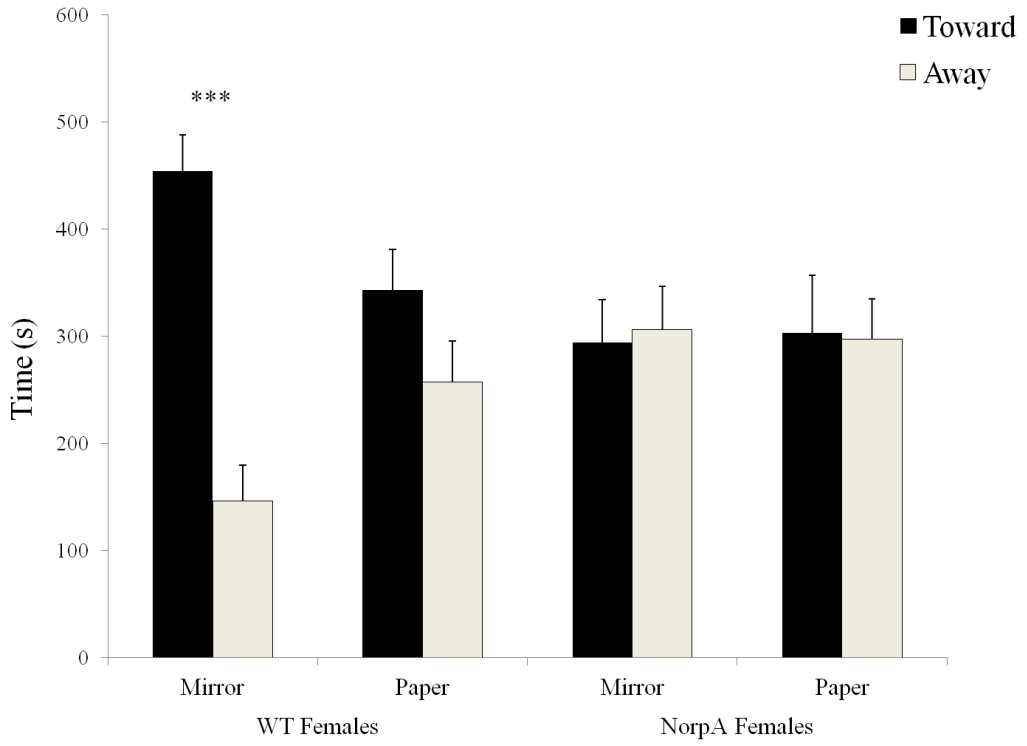


Fig. 3.4. The total time that female wild-type (WT) and NorpA fruit flies spent oriented towards or away from the stimulus during 10 min of observation. Bars depict mean ± SEM; n=40. Female WT fruit flies spent significantly more time oriented towards the mirror compared to away from the mirror (***$p<0.0001$).

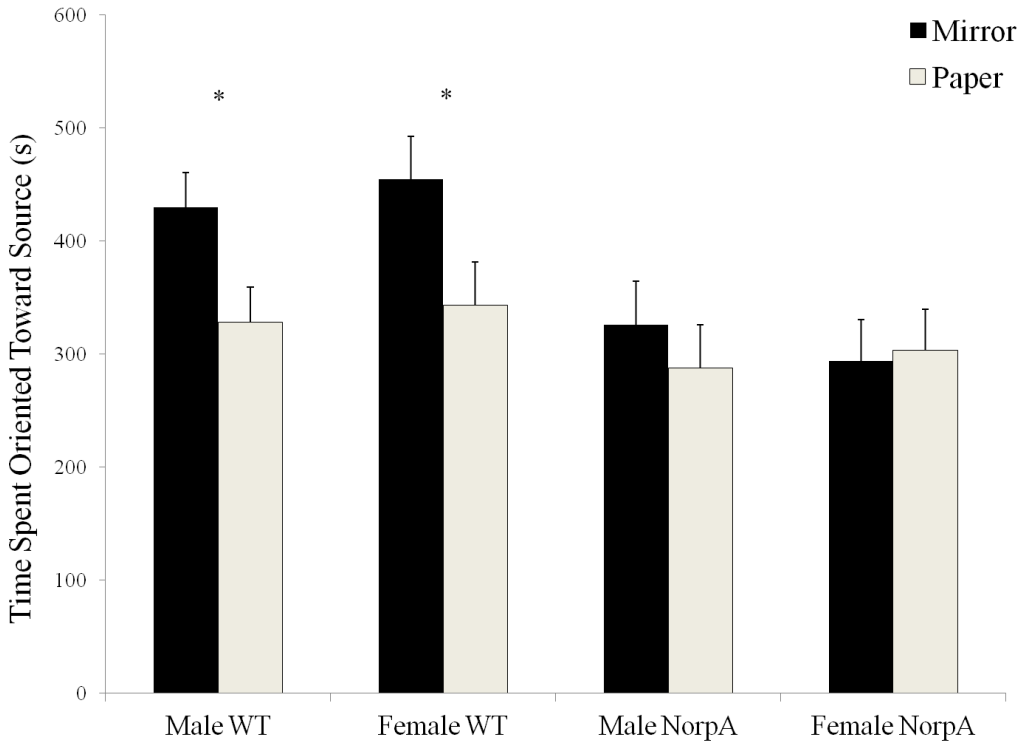
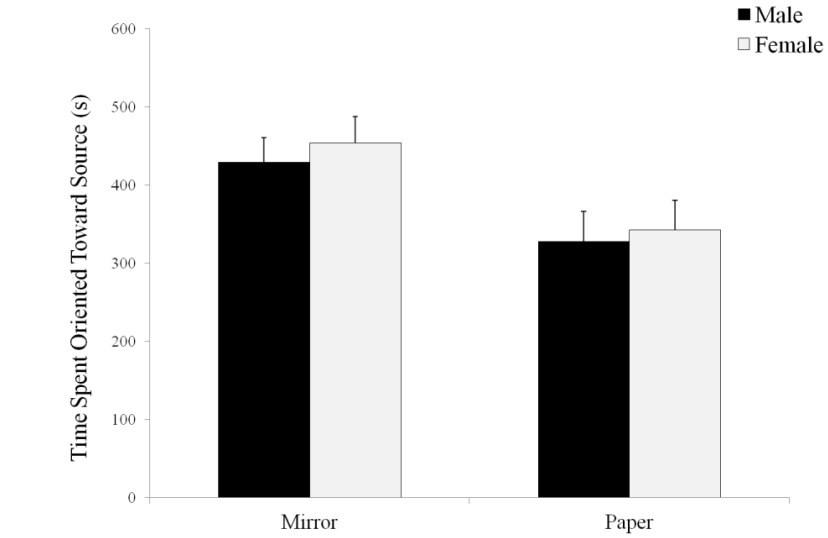


Fig 3.5. The amount of time each fruit fly group spent oriented towards the mirror compared to the amount of time spent oriented towards the paper. Bars depicts mean ± SEM; n=40. Both male and female wild-type (WT) fruit flies spent significantly more time oriented towards the mirror compared to the amount of time spent oriented towards the paper (*$p<0.05$). Neither male nor female NorpA fruit flies spent more time oriented towards the mirror or the paper.

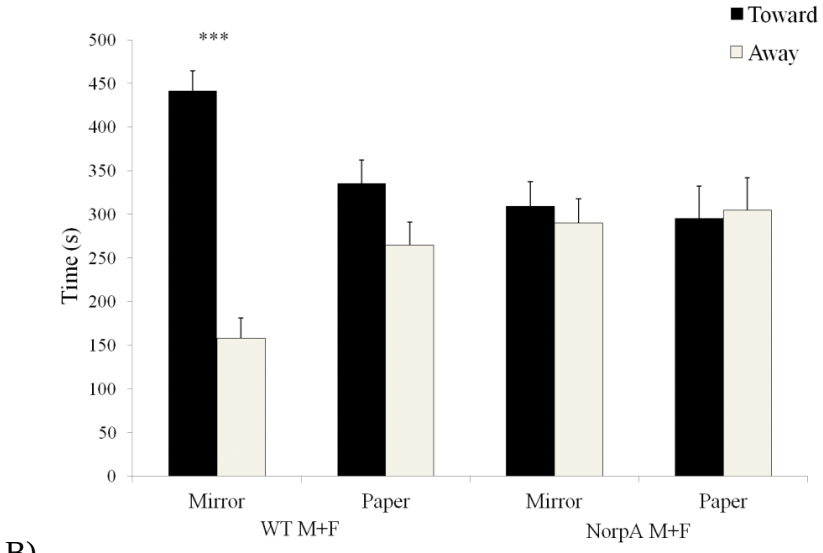
The amount of time that each group spent oriented towards the mirror compared to the amount of time they spent oriented towards the paper was also examined. Both male and female WT fruit flies spent more time oriented towards the mirror compared to the amount of time they spent oriented towards the paper (Fig 3.5) (Paired t-test, $p<0.05$). For NorpA fruit flies, there were no significant differences between the time oriented towards the mirror vs. paper for either males or females.

Since both male and female WT fruit flies oriented significantly longer towards the mirror, a comparison was made between them. Fig 3.6A depicts the amount of time male WT fruit flies spent oriented towards the source (mirror) compared to the amount of time female WT fruit flies spent oriented towards the source. There was no statistical difference between male and female WT fruit flies. Fig 3.6B shows the amount of time male and female (grouped together) WT and NorpA fruit flies spent oriented towards or away from the source of the stimulus. Only WT fruit flies spent more time oriented towards the mirror compared to away from the mirror (Paired t-test, $p<0.0001$). WT fruit flies did not exhibit differences in orientation with respect to the paper. NorpA fruit flies did not show any significant differences in orientation with respect to either the paper or the mirror. Because there was no statistical difference between male and female fruit flies, only male fruit flies were tested following this experiment.

In a second set of trials, attraction to reflection was examined in a cubed observation arena, where two walls were mirrored and two walls were painted white. This effectively divided the arena into reflective and non-reflective environments. The amount of time fruit flies spent in one environment versus the other was calculated. Wild-type fruit flies spent significantly more time in the reflective environment versus the non-.



A)



B)

Fig 3.6. The amount of time male and female WT and NorpA fruit flies spent oriented towards the source of the stimulus during 10 min of observation. A) The amount of time male and female fruit flies spent oriented towards either the paper or the mirror. Bars depict mean ± SEM; n=40. There was no significant difference between male and female WT fruit flies. B) The amount of time male and female (M+F) (grouped together) WT and NorpA fruit flies spent oriented towards or away from the source. Bars depict mean ± SEM; n=80. Only WT fruit flies oriented towards the mirror significantly more than away from the mirror (***$p<0.0001$). Neither group showed a preference for nor avoidance of the paper and NorpA fruit flies showed no preference for or avoidance of the mirror.

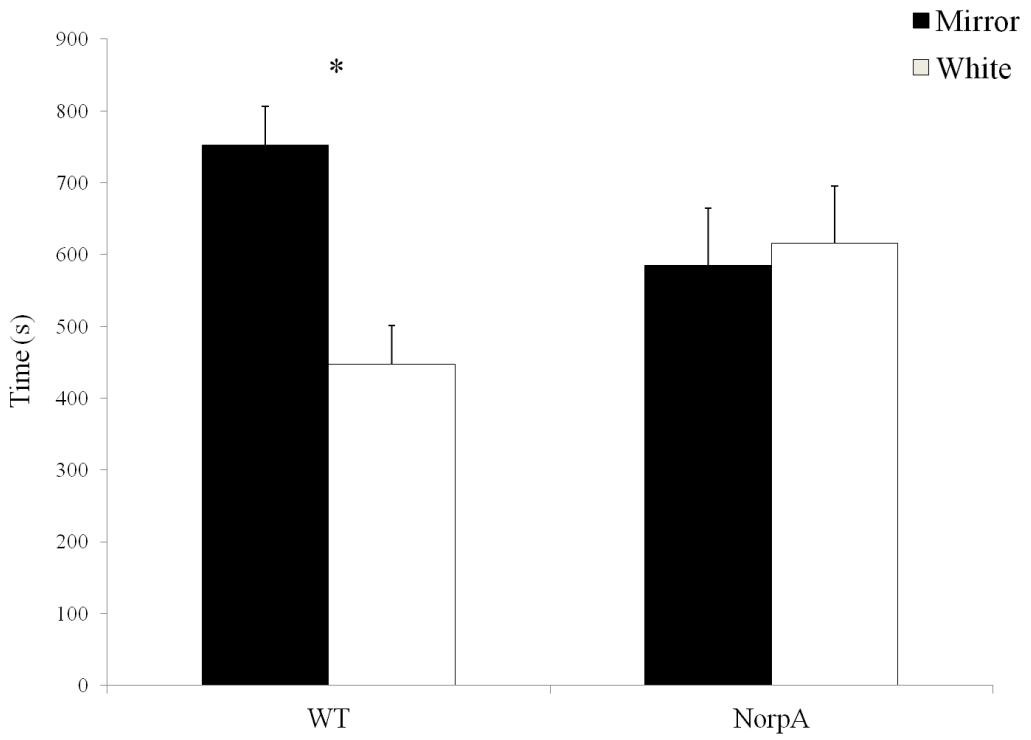


Fig 3.7. The amount of time wild-type (WT) and NorpA fruit flies spent on mirrored versus white walls during 20 min of observation. Bars depict mean ± SEM; n=20. WT flies spent significantly more time on mirrored walls compared to white walls (*$p<0.05$), while NorpA flies showed no preference.

reflective environment (Fig 3.7) (Paired t-test, $p<0.05$). NorpA fruit flies showed no preferences for white or mirrored walls

The data obtained from the Petri dish arena and cubed observation arena indicated that wild type flies are attracted to reflection. The absence of such attraction in NorpA P24 flies indicates that visual inputs are necessary for attraction to mirrors. Given these results, the next step was to characterize more completely how a reflective environment alters various behaviours exhibited by adult *D. melanogaster*.

3.04.3: *Behaviours in a Reflective Environment and Effects of Isolation*

Since social isolation alters responses of crayfish to reflective environments (Drozdz et al., 2006; May & Mercier, 2006), experiments aimed at characterizing responses to reflection in *D. melanogaster* were performed using wild-type male fruit flies that were reared either in isolation or in a social stock vial. Fruit flies were observed individually in a cubed arena where two walls were mirrored and two walls were transparent glass. The amount of time the fruit flies spent on the reflective walls compared to the glass walls was calculated, and so was the frequency of any behaviours performed on any wall. Each fruit fly was observed and videotaped for 20 min.

The amount of time that isolated fruit flies spent on each surface (mirrored, clear glass or the top and bottom of the arena) varied significantly (Fig 3.8) (ANOVA, $P<0.05$). Isolated fruit flies spent significantly more time on mirrored walls compared to the top or bottom of the arena (Tukey HSD, $p<0.05$). Socialized fruit flies showed no preference for any surface.

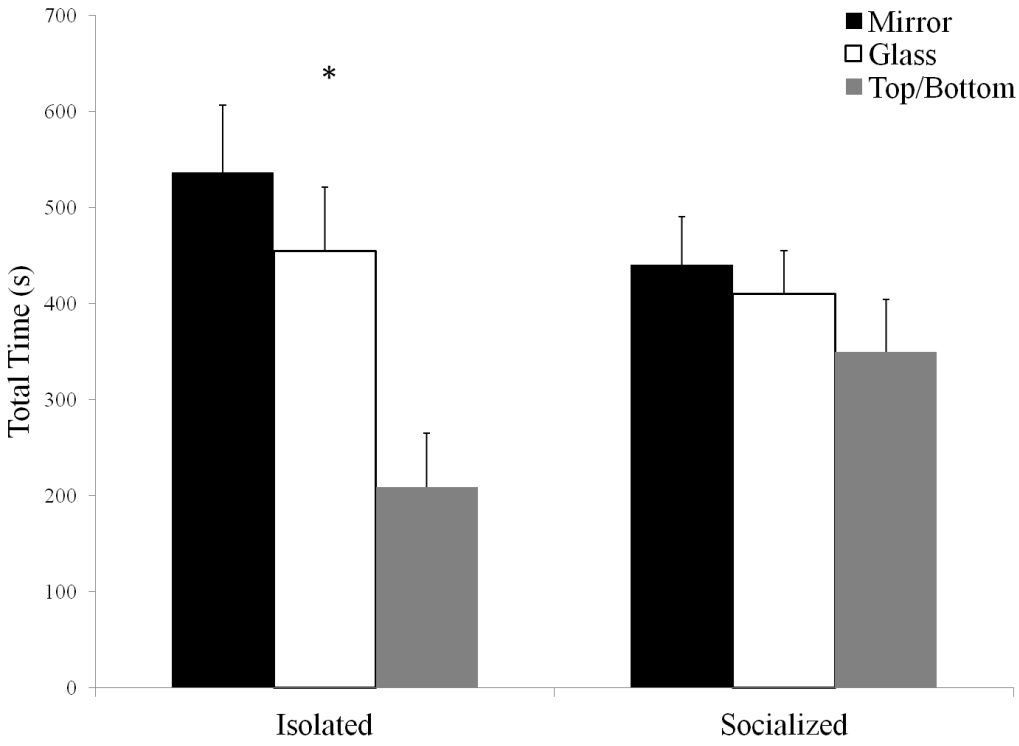
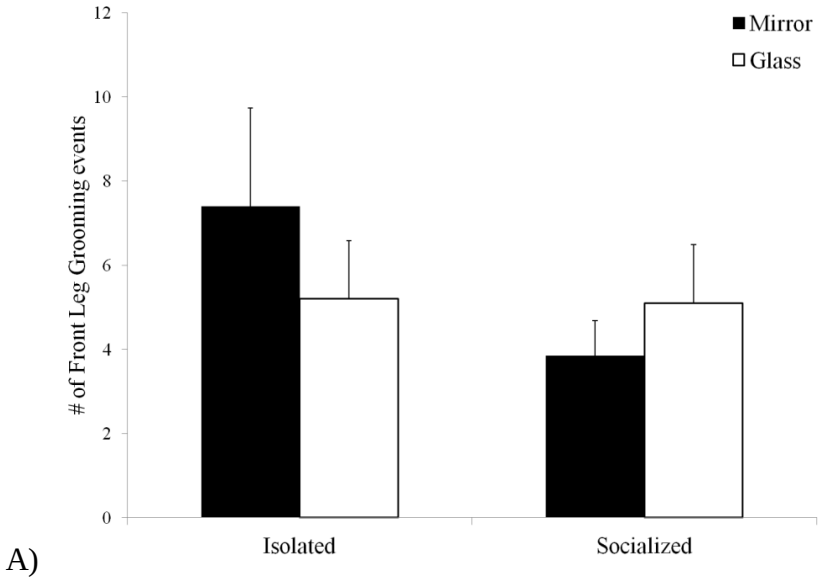
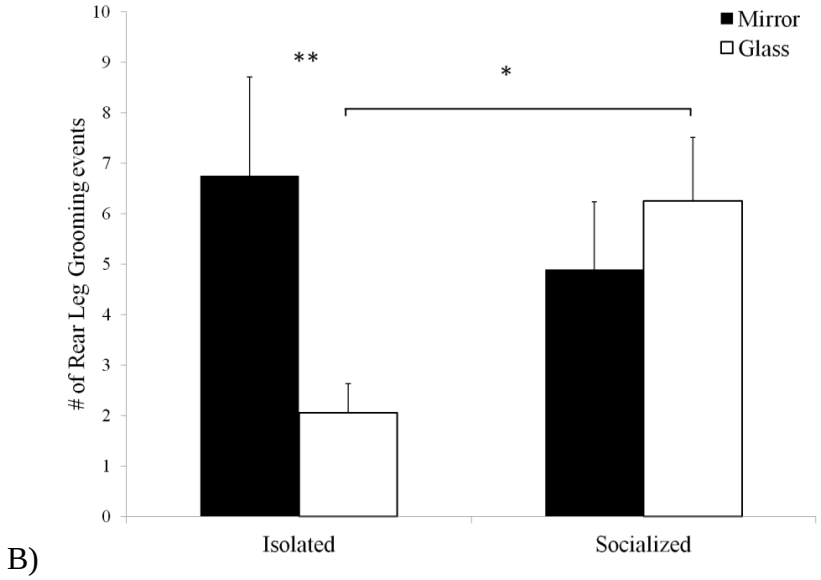


Fig 3.8. The amount of time isolated and socialized wild-type fruit flies spent on each surface (mirrored walls, clear glass walls and top or bottom of chamber), during 20 min of observation. Bars depict mean ± SEM; n=20. The amount of time isolated fruit flies spent on each surface varied significantly (*$P<0.05$). Socialized fruit flies did not demonstrate a preference for any surface.



A)



B)

Fig 3.9. The number of grooming events during 20 min of observation. Bars depict mean ± SEM; n=20. A) There was no difference between the number of front leg grooming events performed on the mirror side compared to the clear glass side, for either group. There was also no difference between isolated and socialized fruit flies with respect to the number of front leg grooming events performed. B) Isolated fruit flies performed more rear leg grooming on the mirrored side of the arena compared to the clear glass side (**$p<0.01$). Socialized fruit flies performed more rear leg grooming on the clear glass side of the arena compared to the mirrored side (*$p<0.05$).

Grooming was a common behaviour performed by fruit flies in the observation arena. Fruit flies performed both front leg grooming and rear leg grooming. Neither socialized nor isolated fruit flies showed any significant difference between the number of front leg grooming events performed on the reflective and clear glass sides of the arena (Wilcoxon Signed-Ranks) (Fig. 3.9a). Isolated fruit flies, however, performed more rear leg grooming events on the reflective side compared to the side with clear glass (Wilcoxon Signed-Ranks, $p<0.01$). Socialized fruit flies performed more rear leg grooming events on the clear glass side of the arena than did isolated fruit flies (Wilcoxon Signed-Ranks, $p<0.05$).

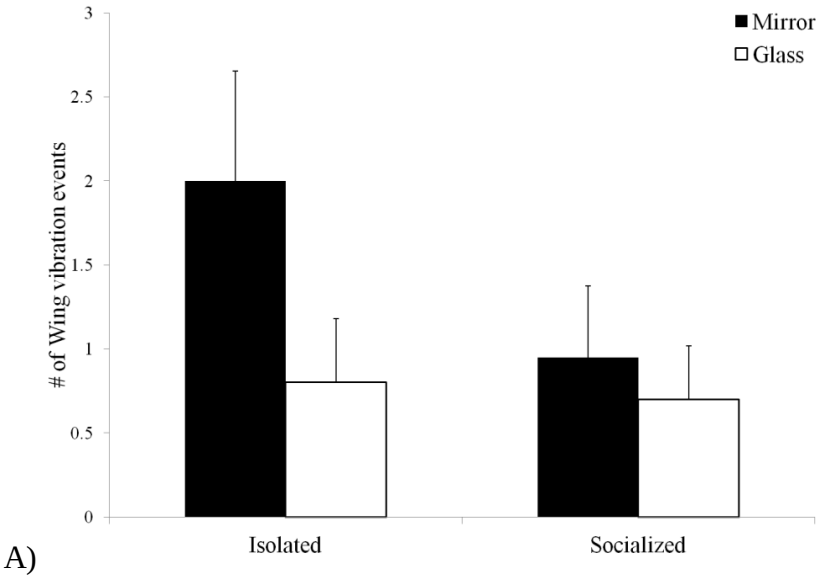
Fruit fly groups performed two distinct wing movements during observation in the test arena. Wing vibration, the vertical vibration of a single wing (Ewing & Bennet-Clark, 1968), was observed in both environments and was performed by both isolated and socialized fruit flies. Isolated fruit flies appeared to exhibit wing vibration more frequently on the mirrored walls compared to the clear glass, but although the data approached statistical significance, the difference was not statistically significant (Wilcoxon Signed-Ranks; $P=0.09$; Fig 3.10a). Socialized flies showed no significant difference for wing vibration between reflective and clear glass surfaces (Wilcoxon Signed-Ranks; $P=0.55$; Fig 3.10a). There was also no significant difference between the number of wing vibrations performed when comparing isolated fruit flies to socialized fruit flies.

Wing scissoring, when the two wings move in opposite directions and pass across each other, was also observed in the test arena. Socialized fruit flies performed more

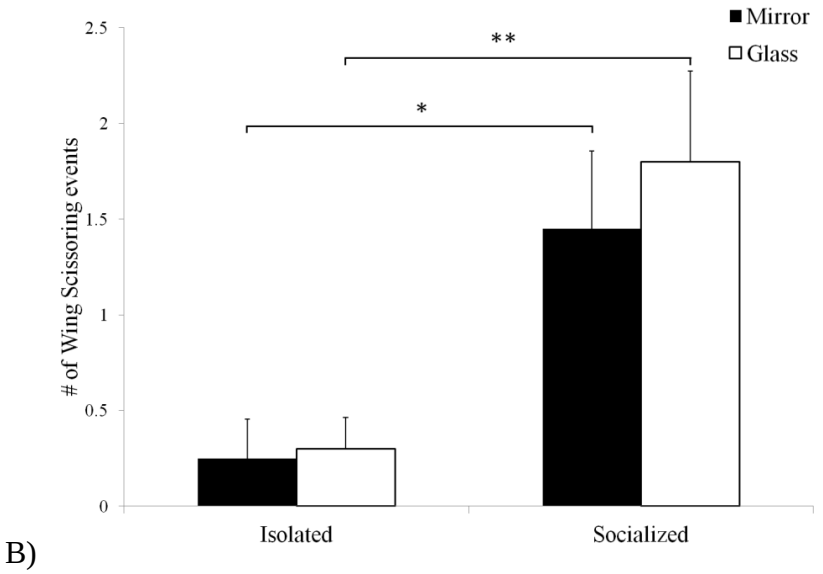
wing scissoring than their isolated counterparts on both the mirrored walls (Mann-Whitney, $p<0.05$) and clear glass walls (Mann-Whitney, $p<0.005$) (Fig 3.10b).

Proboscis extensions were performed by both isolated and socialized fruit flies on both sides of the test arena (Fig 3.11). The data suggested that isolated fruit flies performed more proboscis extensions on the mirrored side of the arena than the non-mirrored side, and although the data approached statistical significance, there was no significant difference (Wilcoxon Signed-Ranks, $p=0.07$). The data were highly variable, and a histogram is provided to illustrate the distributions (Appendix II). There were also no significant differences between the number of proboscis extensions performed by socialized fruit flies on either side of the arena, nor were there any significant differences between the number of proboscis extensions for isolated and socialized groups.

Fruit flies also flexed (bent) the abdomen, touching the end to the substrate, to perform abdominal tapping. Isolated fruit flies performed more abdominal tapping than socialized fruit flies, on both mirrored and non-mirrored walls (Mann Whitney, $p<0.05$) (Fig 3.12).



A)



B)

Fig 3.10. The frequency of wing movements performed by isolated and socialized fruit flies during 20 min of observation. Bars depict mean ± SEM; n=20. A) There was no statistical difference in the number of wing vibrations performed by isolated and socialized fruit flies in either environment of the arena. B) Socialized fruit flies performed more wing scissoring on both the mirrored walls (*$p<0.05$) and clear glass walls (**$p<0.005$) when compared to isolated fruit flies.

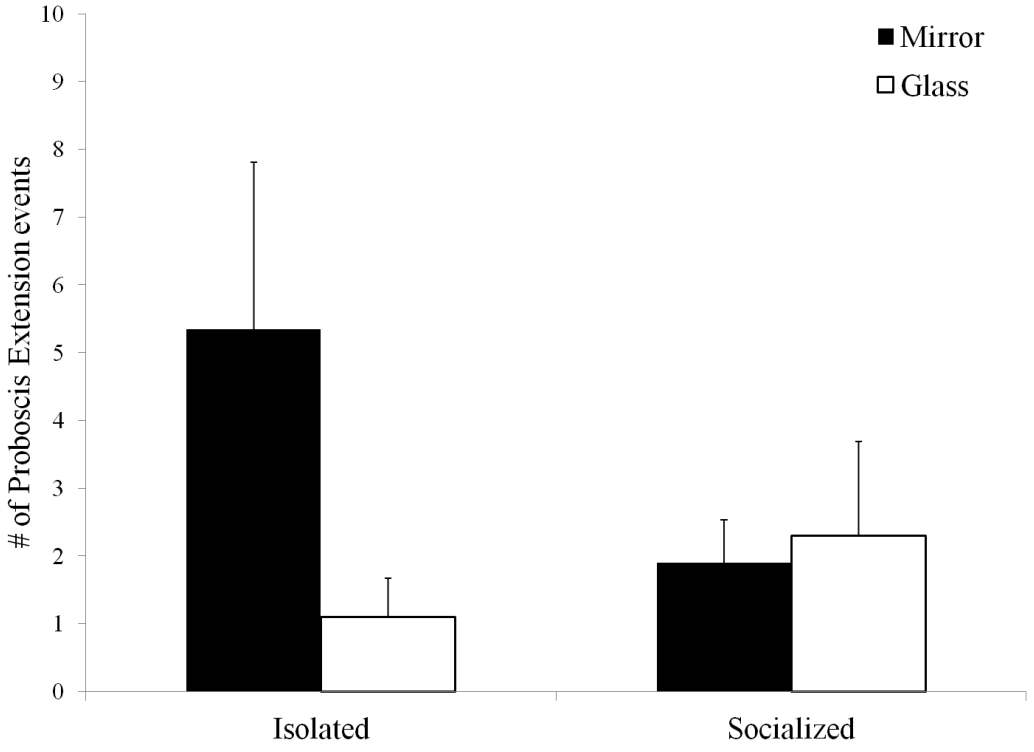


Fig 3.11. The number of proboscis extension events performed by fruit flies during 20 min of observation. Bars depict mean ± SEM; n = 20. There was no statistical difference in the number of proboscis extensions performed by either isolated or socialized fruit flies on either side of the test arena. There were also no differences found between socialized and isolated fruit flies.

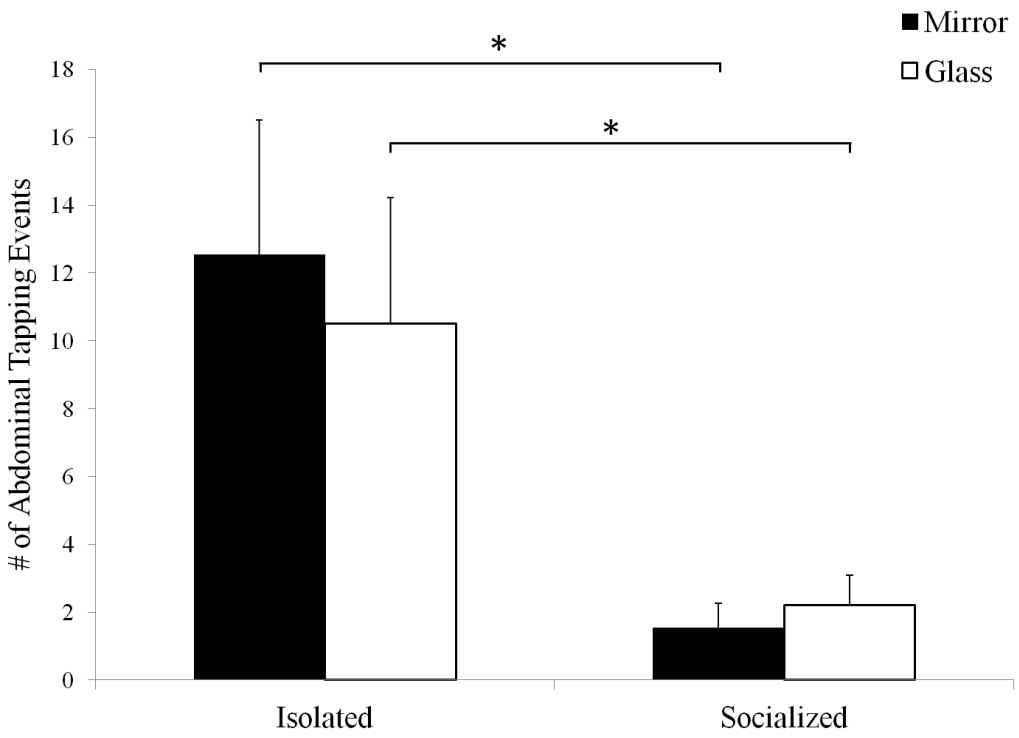


Fig. 3.12. The number of abdominal tapping events performed by isolated and socialized fruit flies during 20 min of observation. Bars depict mean ± SEM; n = 20. Isolated flies performed more abdominal tapping than socialized flies on both sides of the chamber (* $p<0.05$).

3.05: DISCUSSION

The present study demonstrates, for the first time, that an insect, in this case *Drosophila melanogaster*, is attracted to reflection. This study also characterizes behavioural responses to reflection and demonstrates that responses to reflection depend on prior social experience. In a shallow experimental chamber designed to allow flies to orient only toward or away from a mirror, fruit flies spent more time oriented toward the reflection. In a larger cubical chamber, flies spent more time in a reflective environment compared to a non-reflective environment when given a choice between the two. These observations indicate that visual cues are sufficient to alter behaviour in the fruit fly.

NorpA P24 flies are genetically altered flies that are rendered blind by mutation. The gene NorpA encodes for β-PLC, which is expressed in the rhabdomeres of the fly eye (Bloomquist et al., 1988). When light enters the compound eye of a fruit fly, rhodopsin is converted to metarhodopsin via a phototranduction cascade that involves PLC. PLC is necessary for phototransduction, and when inactivated, as it is in NorpA flies, the pathway is blocked and there is a build up of rhodopsin (Pearn et al., 1996). This mutation causes complete inability to respond to light, as demonstrated with an electroretinogram (Fig 3.02). These mutants were chosen as a control for visual cues in the present study, and the results indicate that NorpA flies do not respond to visual cues. These experiments confirm that there was nothing attractive about the mirrors used other than visual stimuli.

Adult male *D. melanogster* exhibited two grooming behaviours in the test chamber, and there was some tendency for socialized flies to behave differently than

isolated flies. Socialized fruit flies, reared with others, performed more rear leg grooming on the non-reflective side of the chamber, compared to isolated flies, but there were no differences in rear leg grooming between socialized and isolated flies on the reflective side. Nonetheless, this observation suggests that socialized flies may perform more grooming overall than do isolated flies. However, the frequency of front grooming did not differ significantly between the two groups on either reflective or non-reflective surfaces. Thus, isolation did not appear to have a dramatic effect on grooming behaviours overall.

Within the test chamber, flies also exhibited behaviours that either are or could be associated with courtship. Behaviours that have been listed as components of courtship in *D. melanogaster* include licking (described here as proboscis extension), wing vibration and wing scissoring (Ewing & Benet-Clark, 1968; Hegde & Krishna, 1997; Yamamoto & Koganezawa, 2013). Abdominal tapping, observed here in both socialized and isolated flies, resembles attempted copulation (Sokolowski, 2001). Thus, three or perhaps four behaviours exhibited in the test chamber resemble aspects of courtship activity. Isolated fruit flies performed more abdominal tapping and more proboscis extension than socialized flies, regardless of the environment. These observations gave the appearance that isolated flies tended to exhibit more courtship-related behaviours in the test chamber than did socialized flies. However, socialized flies performed more wing scissoring than did isolated flies, regardless of environment. Thus, some evidence suggests that isolated males exhibit courtship-related behaviours more frequently overall than do socialized male, but other evidence does not.

One obvious question is whether the frequency of courtship-associated behaviours is elicited or increased by reflection and, if so, whether such effects are influenced by isolation or socialization. In fact, there were no statistically significant differences in the number of occurrences of any of the four behaviours listed above as "courtship-related" between reflective and non-reflective surfaces, and this was true for both isolated and socialized flies. Nonetheless, there were some trends in the data suggesting differences between behaviours on the reflective and non-reflective walls of the test chamber. Although not statistically significant, isolated fruit flies tended to perform more proboscis extensions ($p = 0.07$) and wing vibrations ($p=0.09$) on the mirrored side of the chamber compared to the clear glass side. Since these trends approached statistical significance, they give the impression that isolated (naïve) adult males may exhibit more courtship-related activities in response to reflection. No such trends were observed for socialized flies. The absence of statistically significant differences between courtship-related behaviours on reflective and non-reflective sides indicates that visual inputs provided by reflection fail to surpass the threshold for eliciting courtship activity by themselves. The trends in the data suggest that visual inputs from reflection, however, may be approaching such a threshold in isolated flies, but not in socialized flies.

Previous research into courtship behaviour has shown that fruit flies will court in the absence of visual and chemosensory input (Tompkins et al., 1983) and in the absence of tactile information (Gailey, 1986). In fact, no single sensory cue is required for courtship to occur, but the absence of all three (visual, chemosensory and tactile) senses abolishes courtship behaviour (Tompkins et al., 1983; Gailey, 1986). When male fruit flies are reared in social isolation and, thus, are sexually naïve, they spend more time

courting prior to copulation compared to sexually experienced males (Siegel & Hall, 1979; Dukas, 2005). This effect of isolation may explain the trends observed for isolated male flies in the present study with respect to courtship-related behaviours. Such courtship-related behaviours might occur more reliably if appropriate chemosensory cues are combined with reflected visual inputs.

It is also possible that the isolated male fruit flies do not perceive the mirror image as a female but as a male fly. Svetec and Ferveur (2005) demonstrated that socially naïve male fruit flies are more likely to court males than are sexually experienced flies, and that mutant flies incapable of detecting pheromones are also more likely to court males, than are wild-type flies. Further study using males reared only with other males might help identify the developmental mechanisms that enable males to identify females and preferentially court with them. Rearing males exclusively with males would provide exposure to other flies and social experiences but without exposure to female pheromones.

Fruit flies that are reared in isolation are more aggressive than socially reared flies (Hoffmann, 1990; Johnson et al., 2009). Aggressive male fruit flies exhibit tussling and lunging behaviours towards other male fruit flies (Hoffmann, 1987; Yurkovic et al., 2006). Surprisingly, however, no aggressive behaviours or behaviours that mimicked aggressive activities described by others were exhibited by either socialized or isolated fruit flies in the present study. This suggests that visual cues provided by a reflective environment are not sufficient to elicit aggressive behaviours. The extent to which vision, taction and chemosensory cues play a role in aggression is not entirely known. It is possible that the visual cues provided by the mirror are not sufficient or are inappropriate

to elicit aggression in fruit flies. Visually impaired fruit flies lacking the white gene exhibit a reduction in aggression, however, this may be due to the lack of this functioning gene in the central nervous system (Hoyer et al., 2008). Pheromones, such as 11-cis-vaccenyl acetate (cVA), are known to promote aggression (Wang & Anderson, 2010). Perhaps a combination of these cues is needed to elicit the complete suite of behaviours that occur during fighting.

In conclusion, this study has shown that the fruit fly, *Drosophila melanogaster*, is attracted to reflection. This is the first demonstration of responsiveness to reflection in an insect species. Flies reared in social and isolated conditions exhibit some differences in behaviour, but such differences were largely independent of reflection. Future experiments could examine interactions between reflection and non-visual inputs, such as pheromones.

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CHAPTER 4:

RESPONSES TO REFLECTION IN *DROSOPHILA MELANOGASTER* ARE ALTERED BY MALE AND FEMALE PHEROMONES

4.01: ABSTRACT

This study investigated whether visual and chemical inputs interact to modulate behaviour in *Drosophila melanogaster*. The pheromones (Z,Z) 7,11-heptacosadiene (7,11-HD) and 11-cis vaccenyl acetate (cVA) were applied to both isolated and socialized male fruit flies to investigate how pheromones might modulate their responses to reflection. It was predicted that the male pheromone would elicit aggressive behaviours on reflective surfaces and that the female pheromone would elicit courtship behaviours on reflective surfaces. Male fruit flies were videotaped in an observation chamber constructed of two mirrored walls and two glass walls, and the behaviours performed on the reflective side of the chamber were compared to those performed on the non-reflective side. Socialized males treated with cVA, the predominant hydrocarbon found on male fruit flies, performed both more front leg grooming and wing vibration on the mirrored side of the chamber than on the non-reflective side. They also performed more wing scissoring and proboscis extensions on the non-reflective side than on the reflective side. Isolated males treated with cVA performed more wing scissoring on the mirrored side than on the non-reflective side and more abdominal tapping on the non-reflective side than on the reflective side. Male fruit flies treated with 7,11-HD, the predominant pheromone found on female fruit flies, spent more time in the reflective environment compared to the non-reflective environment but performed significantly fewer behaviours overall, regardless of environment. The data indicate that responses to reflection are modified by pheromones, but the effects were not as predicted.

4.02: INTRODUCTION

Fruit fly behaviour encompasses an array of activities, such as feeding, grooming, fighting, courtship and mating. Behaviour is dependent on sensory feedback, physiological state and previous experience. Most behaviour is dependent on sensory cues from the environment. Fruit flies rely mainly on visual, auditory, chemosensory and tactile sensory modalities, and less on graviperception, thermoreception and nociception. Almost half of the fruit fly brain is devoted to the optic lobes, and vision is thought to be the foremost factor that drives fruit fly behaviour (Fischbach & Dittrich, 1989).

Previous research into visually mediated behaviour in *Drosophila* focuses on flying and food or odour detection. Fruit flies must alter their behaviour based on visual input. For example, flying fruit flies are attracted to long vertical objects and avoid small spots in their visual field, as these stimuli likely represent food and prey, respectively (Maimon et al., 2008). Further investigation into how flies distinguish between predators, prey and conspecifics was conducted using a novel approach. Zabala et al. (2012) designed a fruit fly robot to investigate, among other things, fly-fly interactions. They found that regressive motion (movement from back to front) caused the fruit fly stop or cease movement, whereas progressive movement of the robot (front to back) did not. Cessation of movement in the flies prevented collisions with the robot fly. This recent research paper is the first to investigate the ability of visual cues from conspecifics to modify behaviour.

Previous work in this lab, reported here in Chapter 3, has shown that fruit flies are attracted to their mirror image and perform some behaviours that appear to be related to conspecific interactions. In addition, some of the behaviours exhibited by fruit flies in those experiments were dependent on social experience. Adult males that were reared in isolation performed more abdominal tapping and proboscis extension than did adult males reared in a social environment. Socialized males performed more wing scissoring and rear leg grooming than did isolated males. Although fruit flies reared in isolation are more aggressive than socially reared flies (Hoffman, 1990; Johnson et al., 2009), no signs of aggression were observed in response to reflection (Chapter 3). Moreover, some courtship-related behaviours were observed in the test chamber, reflection had no statistically significant effect on the frequency of such behaviours (Chapter 3). These observations suggest that visual inputs provided by reflection are not sufficient to elicit aggressive or courtship behaviours, and that other sensory inputs are necessary to elicit such responses. The focus of the present study was to determine whether combining chemical cues with the visual inputs provided by a reflective environment would alter behaviour in male fruit flies. The underlying hypothesis investigated is that the integration of inputs from two sensory modalities is more effective at eliciting courtship behaviours than is input from one sensory modality. The present work addresses this hypothesis by determining whether or not combining pheromones with the visual input provided by reflection is sufficient to elicit courtship-related behaviours in adult male fruit flies.

Chemical cues play an important role in fruit fly behaviour and interactions (Wang & Anderson, 2010). Both male and female flies have pheromones located on the

cuticle, and these pheromones are referred to as cuticular hydrocarbons (Ferveur, 2005). These pheromones are sexually dimorphic and have opposing effects on both male and female fruit flies. The predominant female pheromone, (Z,Z)-7,11-heptacosadiene (7,11-HD) (Jallon 1984; Ferveur, 2010) has been shown to evoke courtship in male fruit flies (Billeter, et al., 2009). Previous work has shown that each of three senses (vision, taction and chemosensation) is sufficient to elicit courtship behaviour by itself, but none of these senses is absolutely necessary, provided that at least one of the other senses is active (Tompkins et al., 1983; Gailey, 1986). The relative contribution of each sense in eliciting courtship varies with different species of fruit fly (Cobb & Ferveur, 1995). Nonetheless, pheromones are the predominant signal that male fruit flies rely on to trigger courtship (Dickson, 2008).

Courtship behaviours in fruit flies are heavily stereotyped and have been thoroughly described (Sokolowski, 2001). Male *D. melanogaster* begin courtship with females by orienting themselves into position and tapping the female's abdomen. The male "sings" to the female by extending a single wing and vibrating it to produce sound. This is followed by licking the abdomen and then an attempt at copulation. Several behaviours observed in the chamber used for testing effects of reflection (wing scissoring, wing vibration, proboscis extension and abdominal tapping) mimic these courtship behaviours (Chapter 3). Based on the hypothesis stated above, it is predicted that the application of the female pheromone 7, 11-HD onto male fruit flies will increase the frequency of courtship-like behaviours exhibited by isolated fruit flies in a reflective environment. Since socialized fruit flies do not perform these behaviours at a high frequency in a reflective environment (Chapter 3), it is predicted that application of

female pheromones may provide the additional incentive required to elicit courtship behaviours in this group.

The present work also tests the hypothesis that combining chemosensation with visual cues is necessary to trigger aggressive behaviours in fruit flies. The predominant male pheromone, 11-cis-vaccenyl acetate (cVA) promotes aggression in male fruit flies (Wang et al., 2011). Male fruit flies are aggressive and engage in agonistic encounters that include lunging and kicking behaviours against a male opponent, usually in the presence of an incentive such as food or a mate (Chen et al., 2002; Certel et al., 2007). Fights result in a winner and a loser, and after a number of interactions, one member of the pair is deemed dominant and the other is deemed subordinate. Application of 500 μg of cVA to filter paper in an observation chamber has been shown to elicit such behaviours in males, even in the presence of females (Wang & Anderson, 2010). Although aggressive behaviours were not observed in previous experiments examining responses of fruit flies to reflection (Chapter 3), other arthroprod species exhibit aggression-associated behaviours in a reflective environment (Dunham et al., 1986; Chapter 2). It was predicted, therefore, that addition of cVA, onto male fruit flies would elicit aggressive behaviours in both isolated and socialized fruit flies exposed to mirrors.

4.03: METHODS

A total of 120 male Canton-S *Drosophila melanogaster* was used for these experiments. Of these, 60 were reared in isolation, and 60 were reared in a community stock vial, as described in Chapter 3. All flies were tested 1-3 days post eclosure and no fly was tested more than once. Data from flies that received no treatment (Chapter 3) were included in figures.

Isolated adult males (N=20) and socialized adult males (N=20) were exposed to 11-cis-vaccenyl acetate (cVA) (Caymen Chemical Co.). Flies were anesthetized using CO_2 before 50 µL of cVA (500 mg/L), dissolved in 95% ethanol, was applied to the dorsal abdomen of the fruit fly using a micropipette while viewing under a dissecting microscope. An additional 20 isolated and 20 socialized fruit flies were anesthetized using CO_2 and 7,11 hexacosadiene (7,11 HD) (Caymen Chemical Co). 50 µL of 7,11 HD (500mg/L), dissolved in 95% ethanol, was applied to the dorsal abdomen using a micropipette. Thus, each fly in the pheromone groups was exposed to 25µg of either male or female pheromone. The remaining 20 isolated and 20 socialized adult male fruit flies were exposed to ethanol. As with the pheromones, 50 µL of 95% ethanol was applied to the abdomen of the fruit fly, while viewing through a microscope.

The pheromones were applied to the abdomen of the fruit flies for a number of reasons. Firstly, it insured that each fly received the same measure of pheromone. The pheromones were not added to the filter paper because it possibly would have attracted the flies away from the walls of the chamber. The pheromones were not applied to the walls because it was impossible to apply the same amount to each wall with consistency.

Applying the pheromone to the fly itself also assisted with cleaning the chamber (with ethanol) to insure it was "pheromone free" between trials.

Each fruit fly was placed into a 1.5mL Eppendorf tube for one hour prior to testing. The tube was capped with cotton batten, moistened with distilled water. After the hour rest, each fly was placed individually into the test arena and filmed for 20 min, using a camcorder (Sony) connected to a PC-compatible computer. The test arena consisted of a cube with two side walls made of clear glass and two side walls made of mirrors (Fig 3.1A). Lighting was provided by a single fluorescent tube light placed directly above the set-up and fluorescent ceiling lights. Behaviours performed on either the clear glass or mirrored walls were counted in addition to the time spent on these walls. Fruit flies spent little time on the floor or top lid of the chamber and, neither the time spent there, nor the behaviours performed there were scored. The test chamber was swabbed with ethanol and allowed to dry between trials. The videos were later viewed on a PC, using Windows Media Player and analysed to determine the amount of time each fruit fly spent on the mirrored versus the glass side of the arena. The frequency of any behaviour performed was also measured (see Table 3.1 for descriptions of behaviours). An F-distribution test was performed and the data were not found to be normally distributed. For this reason, non-parametric statistical tests were performed for data described in this chapter.

4.04: RESULTS

The average time each fruit fly spent on the mirrored side of the arena compared to the clear glass side was determined for each group (Fig 4.1). Socialized fruit flies that were exposed to 7,11-HD spent significantly more time on the mirrored side of the arena compared to the glass side (Wilcoxon Signed-Ranks, $p<0.05$). Isolated fruit flies that were exposed to ethanol and isolated fruit flies that received no treatment also spent more time on the mirrored side of the arena compared to the clear glass side (Wilcoxon Signed-Ranks, $p<0.05$) but socialized flies exposed to ethanol did not.

Grooming was observed in the test arena. Both front leg grooming and rear leg grooming were observed and analyzed. Socialized fruit flies that were exposed to cVA performed more front leg grooming on the mirrored side of the test arena than on the clear glass side (Fig 4.2A) (Wilcoxon Signed-Ranks, $p<0.05$). No other fruit fly group demonstrated any significant differences with respect to front leg grooming on either side of the arena. Isolated fruit flies that were exposed to ethanol and isolated fruit flies that received no treatment both demonstrated more rear leg grooming on the mirrored side of the arena compared to the clear glass side (Fig 4.2B) (Wilcoxon Signed-Ranks, $p<0.005$). No other fruit fly groups showed a preference for rear leg grooming on either side of the test arena.

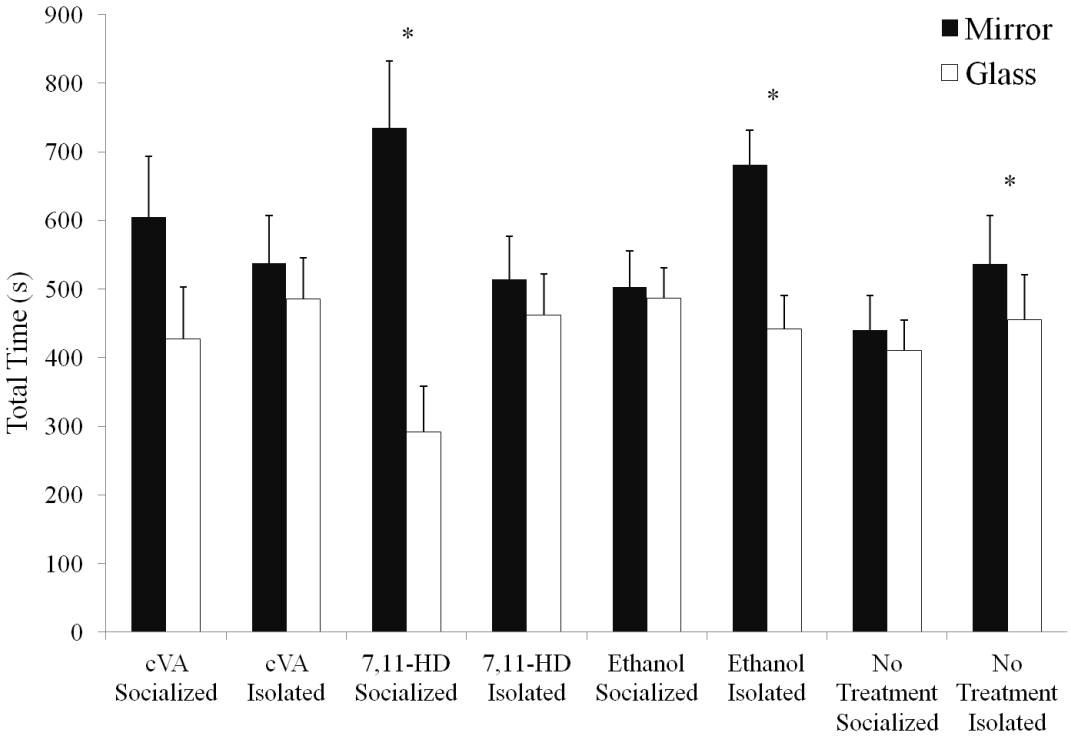
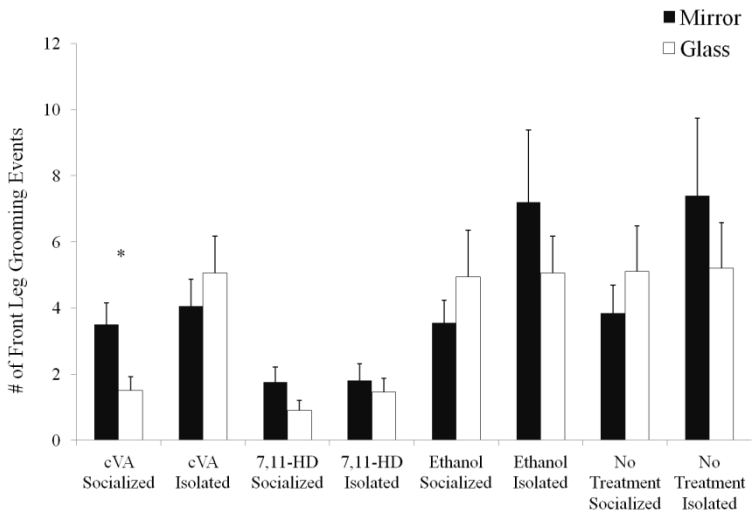
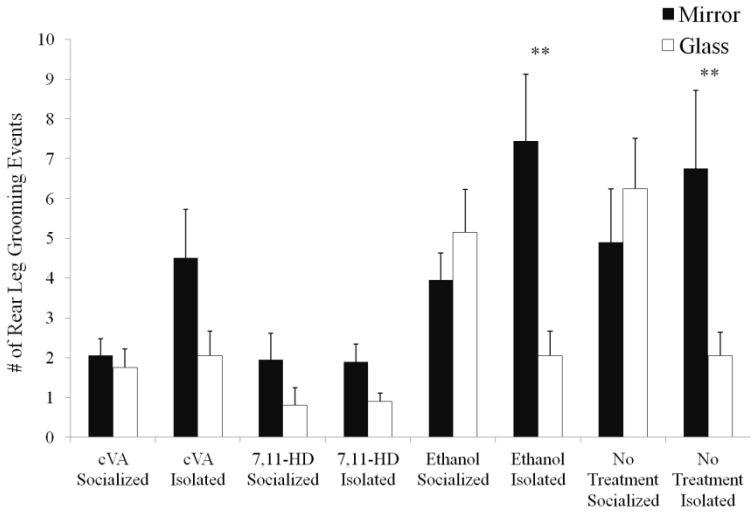


Fig. 4.1. The amount of time each fruit fly group spent on the mirrored walls of the arena compared to the clear glass walls during 20 min of observation. Time spent on the top or bottom of the chamber was not calculated. Bars depict mean ± SEM; n=20. Socialized fruit flies exposed to 7,11-HD spent significantly more time on the mirrored side of the arena compared to the glass side (*$p<0.05$). Isolated fruit flies that were exposed to ethanol and isolated fruit flies that did not receive treatment also spent more time on the mirrored walls of the arena compared to the clear glass walls (*$p<0.05$).



A)

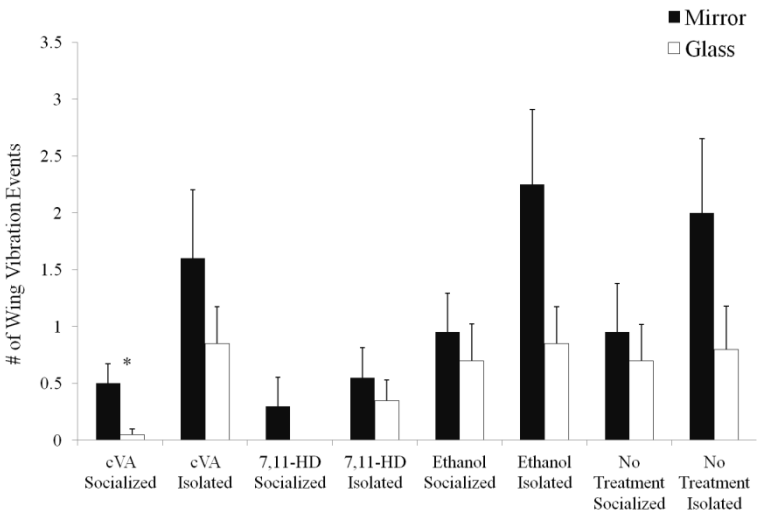


B)

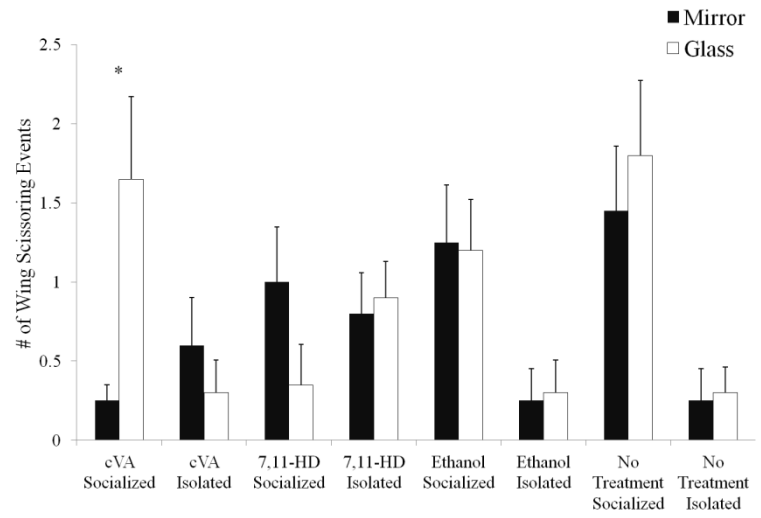
Fig 4.2. The number of grooming events performed by each fruit fly group during 20 min of observation. Bars depict mean ± SEM; n=20. A) Socialized fruit flies that were exposed to cVA performed front leg grooming significantly more frequently on the mirrored side of the arena compared to the glass side (*$p<0.05$). No other group of fruit flies showed a significant difference with respect to front leg grooming. B) Isolated fruit flies that were exposed to ethanol and isolated flies that received no treatment performed more rear leg grooming on the mirrored side of the arena compared to the glass side (**$p<0.005$). No other fruit fly group demonstrated a preference for rear leg grooming on either side of the arena.

Fruit flies also exhibited wing behaviours during observation in the test arena. Socialized fruit flies that were exposed to cVA performed more wing vibration events on the mirrored walls of the arena compared to the clear glass walls (Fig 4.3A; Wilcoxon Signed-Ranks, $p<0.05$). No other fruit fly group showed a preference for wing vibration on either side of the test arena. Socialized fruit flies that were exposed to cVA also performed wing scissoring events more frequently on the clear glass walls of the arena compared to the mirrored walls (Fig 4.3B) (Wilcoxon Signed-Ranks, $p<0.05$). No other fruit fly group demonstrated a statistically significant difference between the number of wing scissoring events performed on the mirrored or clear glass walls of the test arena.

Fruit flies also performed proboscis extensions in the test arena (Fig 4.4). Socialized fruit flies that were exposed to cVA performed more proboscis extensions on the clear glass side of the test arena compared to the mirrored side (Wilcoxon Signed-Ranks, $p<0.05$). The opposite effect occurred in isolated fruit flies exposed to cVA, which performed more proboscis extensions on the mirrored side of the arena compared to the clear glass side (Wilcoxon Signed-Ranks, $p<0.05$). Isolated fruit flies that were exposed to ethanol also performed more proboscis extensions on the mirrored side of the test arena compared to the glass side (Wilcoxon Signed-Ranks, $p<0.05$). No other fruit fly group showed a preference for proboscis extensions on either side of the arena.



A)



B)

Fig 4.3. The frequency of wing behaviour performed by each fruit fly group during 20 min of observation. Bars depict mean ± SEM; n = 20. A) Socialized fruit flies exposed to cVA performed more wing vibrations on the mirrored side of the test arena compared to the clear glass side (*$p < 0.05$). No other fruit fly group showed a preference for wing vibrations on either side of the arena. B) Socialized fruit flies exposed to cVA performed more wing scissoring on the clear glass side of the test arena compared to the mirrored side (*$p<0.05$). No other fruit fly group demonstrated a significant difference in the number of wing scissoring events performed on either side of the arena.

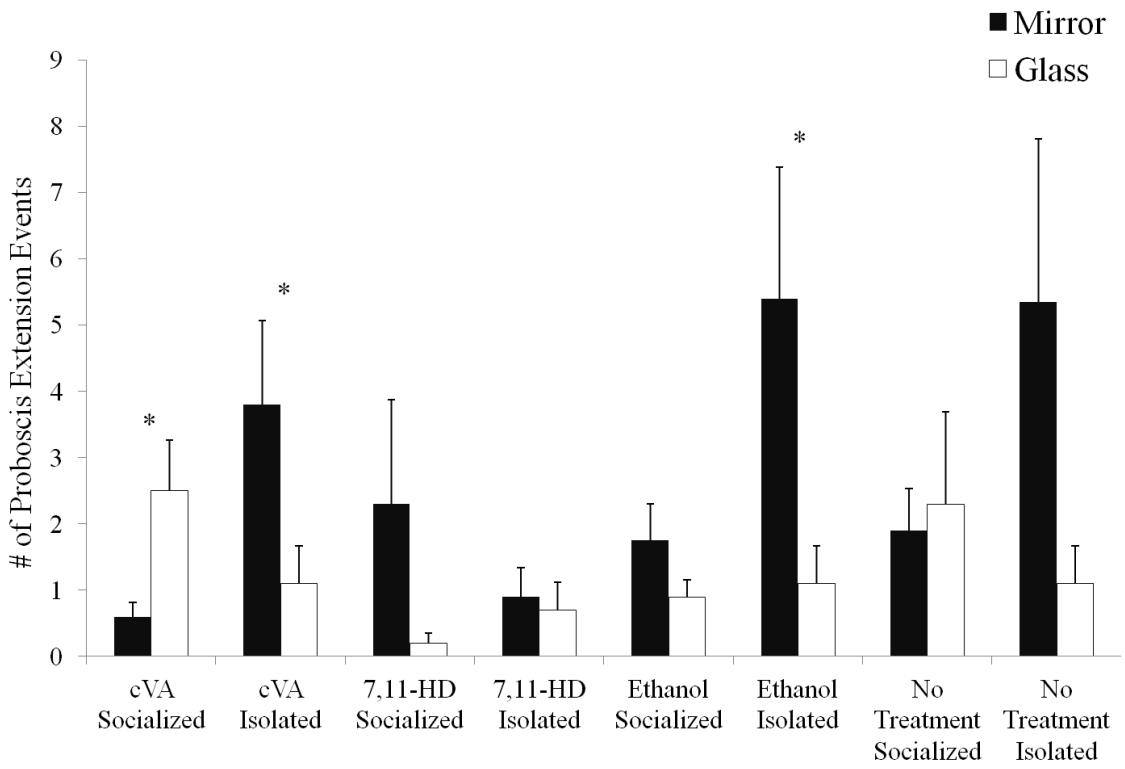


Fig 4.4. The frequency of proboscis extension events performed by each fruit fly group during 20 min of observation. Bars depict mean ± SEM; n = 20. Socialized fruit flies exposed to cVA performed more proboscis extensions on the clear glass side of the arena compared to the mirrored side (*$p<0.05$), whereas isolated fruit flies exposed to cVA performed more proboscis extensions on the mirrored side of the arena compared to the glass side (*$p<0.05$). Isolated fruit flies exposed to ethanol also performed more proboscis extensions on the mirrored side of the arena compared to the clear glass side (*$p<0.05$). No other fruit fly group demonstrated a preference for proboscis extension events on either side of the test arena.

Fruit flies also exhibited abdominal tapping while in the test arena. Isolated fruit flies that were exposed to cVA performed more abdominal tapping on the glass side of the arena compared to the mirrored side (Fig 4.5; Wilcoxon Signed-Ranks, $p<0.05$). No other fruit fly group showed statistically significant differences between the number of abdominal taps performed on the two sides of the test arena.

When comparing the behaviours observed by each fruit fly group, it appears that fruit flies exposed to pheromones performed fewer of the described behaviours overall than did fruit flies exposed to just ethanol. There were no significant differences between the frequency of behaviours of fruit flies exposed to ethanol alone and fruit flies not treated with any chemical substance (Appendix III). The sum of all described behaviours performed on the mirrored side of the test arena during the 20 min observation period was calculated to determine if there was a difference in how active fly groups were (Fig 4.6). Because each behaviour is performed briefly, and behaviours performed repeatedly are counted as individual occurrences, this is a good measure of activity. There was a significant effect of chemical treatment on the mean number of behaviours performed by isolated fruit flies on the mirrored side of the arena (Kruskal-Wallis Test, $p<0.0001$).

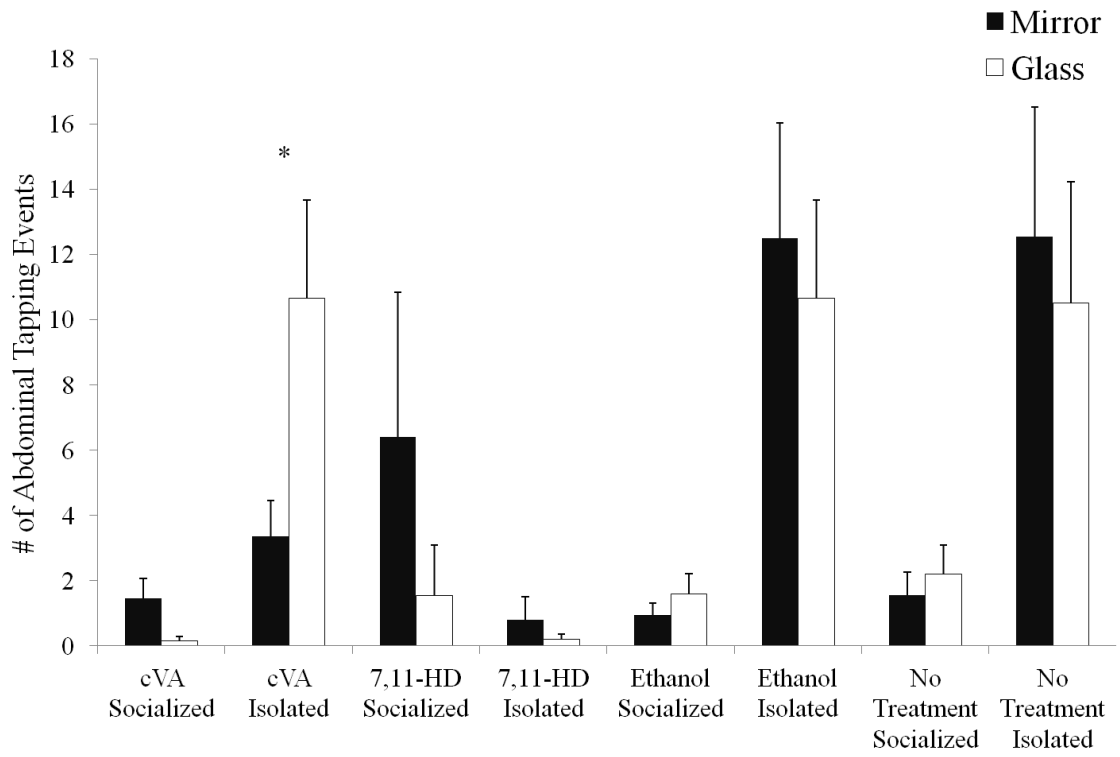


Fig 4.5. The frequency of abdominal tapping events performed by each fruit fly group during 20 min of observation. Bars depict mean ± SEM; n = 20. Isolated fruit flies that were exposed to cVA performed more abdominal tapping on the clear glass side of the test arena compared to the mirrored side (*$p<0.05$). No other fruit fly groups exhibited a difference in the frequency of abdominal tapping performed on either side of the arena.

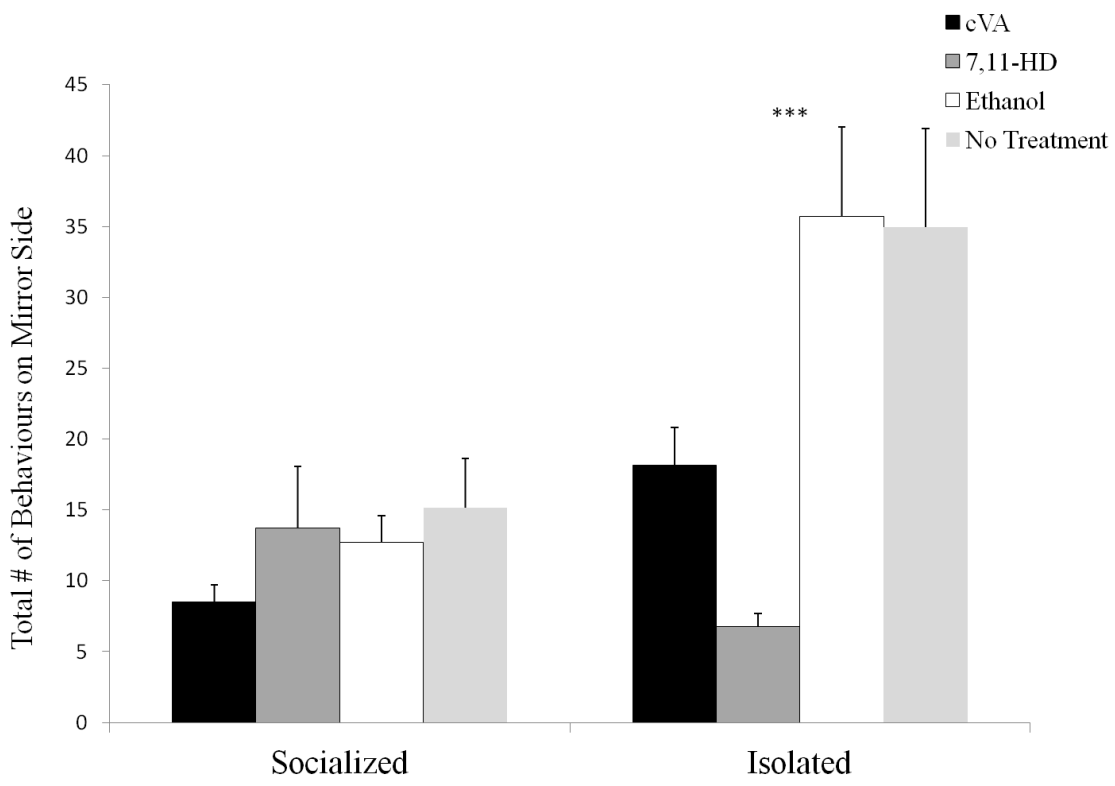


Fig 4.6. The total number of behaviours performed by each fruit fly group on the mirrored side of the test arena during 20 min of observation. Bars depict mean ± SEM; n=20. There was a significant difference in the mean number of behaviours performed by isolated fruit fly groups on the mirrored side of the test arena (***$p<0.0001$) compared to the clear glass side. There was no significant difference in the number of behaviours performed by socialized fruit flies.

4.05: DISCUSSION

The results presented here show that although the pheromones, 7,11-HD and cVA, modified the responses of fruit flies to a reflective environment, they did not conform to the predictions described above (see Introduction). It was predicted that 7,11-HD, the predominant female pheromone, would increase the number of courtship behaviours (wing scissoring, wing vibration, proboscis extension and abdominal tapping; Ewing & Bennet-Clark, 1968; Hegde & Krishna, 1997) on reflective walls of the test chamber. Socialized flies treated with 7,11-HD spent more time on the mirrored side of the chamber compared to the clear glass side, and there was no effect of ethanol on socialized flies. Thus, application of this female pheromone to adult males altered responses to reflection in a manner that suggests attraction to a reflected image. This, however, was the only statistically significant effect of 7,11-HD with respect to reflection. Thus, although combining pheromonal inputs with visual inputs alters responses to reflection, the combination of sensory signals used here was not sufficient to increase the frequency of the courtship behaviours that were examined.

Although 7,11-HD did not increase the expression of courtship behaviours with respect to reflection, it did influence behaviour. The total number of behaviours performed, regardless of reflection, was significantly lower for fruit flies treated with 7,11-HD than for those treated with ethanol alone ($P=0.008$, Mann-Whitney). When all behaviours were combined, isolated flies treated with 7,11-HD performed significantly fewer wing vibrations on mirrored walls compared to control flies treated with ethanol ($P= 0.02$, Mann-Whitney). This finding was unexpected, since 7,11-HD promotes wing vibration in male fruit flies regardless of the sex of fly with which they are paired (Cobb

& Jallon, 1990). Isolated flies also performed significantly fewer proboscis extensions (P=0.004, Mann-Whitney) and less abdominal tapping (P=0.002, Mann-Whitney) on mirrored walls when compared to isolated flies treated with ethanol. Thus, in the experiments reported here, 7,11-HD decreased the occurrence of courtship behaviours in isolated flies, and this result was not expected. No such differences occurred in socialized flies.

It was also predicted that the male pheromone, cVA, would elicit aggressive behaviours (e.g. lunging, kicking and tussling; Certel et al., 2007) in response to reflection. Socialized flies that were treated with cVA did not perform any aggressive behaviours on either side of the test arena. Nonetheless, they performed more front leg grooming and wing vibration on the mirrored walls compared to the clear glass walls. Socialized flies treated with cVA also performed wing scissoring and proboscis extensions on the clear glass walls more frequently than on the mirrored walls. Isolated flies treated with cVA also failed to exhibit aggressive behaviours, but they performed more proboscis extensions on the mirrored walls compared to the clear glass walls and more abdominal tapping on the clear glass walls compared to the mirrored walls. Isolated fruit flies that were used as a control and treated with ethanol spent more time on the mirrored side and performed more rear leg grooming and proboscis extensions on the mirrored walls compared to the clear glass walls. When the total number of behaviours performed was compared between groups, isolated fruit flies treated with ethanol alone performed more behavioural events on the mirrored side of the chamber compared to isolated flies treated with either 7,11-HD or cVA.

Of the two pheromones, 11-cis vaccenyl acetate had the greater effect on both social and isolated fruit flies. cVA has been shown to inhibit courtship at concentrations of 100 ng and higher (Zawistowski & Richmond, 1986). cVA, naturally found on the male fruit fly cuticle, is transferred to the female during mating. This pheromone is thought to inform future potential mates that the female is not a virgin. If given a choice, both inexperienced and experienced male fruit flies are more likely to copulate with virgin females than with mated females (Dukas, 2005). This pheromone also promotes aggression in male fruit flies (Wang & Anderson, 2010; Wang et al., 2011). When 500 μg of cVA was applied to filter paper in an experimental chamber containing two *D. melanogaster* adult males, the number of lunges increased significantly in both members of the pair (Wang & Anderson, 2010). The effects were dose-dependent, and the greatest effect occurred in response to 500 μg of cVA, which was the amount applied to male flies in the present work. Here, pheromone was applied to the abdomen, rather than to the chamber walls or to filter paper, to standardize the amount of pheromone exposure, to avoid attracting the flies away from the walls of the chamber and to assist in cleaning the chamber with ethanol between trials. Nonetheless, the present results raise the question of whether the sex pheromones were effective in activating the appropriate chemoreceptors in the present work. On the other hand, cVA and 7,11-HD are volatile, and the dosage used here should have been sufficient to activate pheromone receptors. Pheromones are detected by receptors in olfactory receptor neurons (ORNs) (Kurtovic et al., 2007). Perhaps an ORN mutant that lacks the ability to perceive pheromones is a more appropriate control. While the dose used here was reported to be optimal in other

experiments (Wang & Anderson, 2010; Wang et al., 2011), a dose-response curve would have been beneficial using this experimental design.

Some observations suggest that the pheromone dosage may have been too high for the method of application used in the present work. Many fruit flies kept relatively still during the 20 minutes of observation. Moreover, six of the twenty socialized fruit flies treated with 7,11-HD appeared to ejaculate on the wall surface, including four on mirrored walls and two on glass walls (data not shown; but photographs are in Fig. 4.7). In the example shown, the fly was stationary for 309 s prior to releasing the substance on the wall and 71 s after release. As this observation was made viewing the films, well after the experiments took place, there was no way to analyze the substance to confirm that it was semen. This observation does suggest, however, that the application of the female pheromone may have surpassed some threshold and overwhelmed some fruit flies.

Fig. 4.7. Frames of video depicting a socialized fruit fly treated with 711-HD ejaculating an unknown substance. The first frame depicts the fly prior to ejaculation and the fly is circled. The second frame depicts the substance when it first appears (arrow) and the third frame depicts the substance after the fly walks away.

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CHAPTER 5

GENERAL DISCUSSION

5.01: GENERAL DISCUSSION

This thesis makes several contributions to knowledge regarding how social experience influences responses to reflection in two invertebrate species. It demonstrates that subordinate crayfish acquire subordinate responses to reflection more quickly when they experience breaks between pairing periods compared to one long pairing period. Spaced pairing also decreased aggression index in subordinate crayfish more rapidly than did massed pairing. These observations support earlier suggestions (May & Mercier, 2006; 2007) that full acquiescence to subordinate status may require more than a single 30 min pairing session, and they also suggest that such acquiescence may be acquired more rapidly when their encounters follow a time schedule similar to traditional training paradigms. The implication of these findings is that dominance rank is learned and requires formation of long-term memory, and that in *P. clarkii*, the involvement of learning appears to be more evident in animals that acquire subordinate status.

This thesis also shows for the first time that fruit flies are attracted to their reflection, and that fruit flies that have been socialized respond to their reflection differently than those reared in isolation. The first ethogram of responses to reflection in fruit flies is reported here (summarized in Table 5.1) and shows that adult males exhibit a combination of grooming-like and courtship-like behaviours in a reflective environment. Socially isolated flies exhibited more rear leg grooming and showed a trend toward exhibiting courtship-like behaviours (wing vibration and proboscis extension) more frequently on a reflective surface, but socialized flies did not.

Table 5.1. Summary of results from Chapters 3 and 4. These results represent an ethogram of behaviours exhibited by male *D. melanogaster* in an experimental chamber designed to examine effects of reflection and the effects of the pheromones cVA and 7,11-HD.

	Time	Front Leg Grooming	Rear Leg Grooming	Wing Vibration	Wing Scissoring	Proboscis Extensions	Abdominal Tapping
Chapter 3							
Isolated	*(M)		*(M)	A		A	
Socialized							
Chapter 4							
Isolated Ethanol	*(M)		*(M)			*(M)	
Socialized Ethanol							
Isolated cVA						*(M)	*(G)
Socialized cVA		*(M)		*(M)	*(G)	*(G)	
Isolated 7,11-HD							
Socialized 7,11-HD	*(M)						

*(M) indicates that the mean was significantly higher on the mirrored walls compared to clear glass walls. *(G) indicates that the mean was significantly higher on the clear glass walls compared to mirrored walls. (A) indicates that the mean approached significance, with a higher mean on the mirrored walls compared to clear glass walls.

Application of male and female pheromones to the abdomen of adult males altered the frequency of grooming and courtship behaviours but did not introduce any new behaviours. The male pheromone (cVA) failed to elicit any aggressive behaviours (e.g. lunging, boxing, etc.), but it suppressed the appearance of greater rear leg grooming in isolated males on the mirror. In socialized flies, cVA caused a preference for front leg grooming and wing vibration on the mirror, and it caused a preference for wing scissoring and proboscis extension on clear glass. The female pheromone (7,11-HD) also suppressed rear leg grooming of isolated flies on the mirror, and it suppressed their preference for the mirror. Both pheromones tended to make adult males less active. The behavioural significance of these effects is not clear.

Learning Dominance Rank

Responses to reflection have previously been examined in mammals but only recently has the ability of invertebrates to respond to reflection been investigated (Kravitz & Huber, 2003). This knowledge has been expanded upon here. I have previously shown that visual cues are sufficient to modify behaviours such as approach (May & Mercier, 2006). Previous research has shown that when two crayfish are placed into an aquarium, one will approach the other and a number of agonistic encounters will ensue (Bovbjerg, 1953; Bruski & Dunham, 1997; Zulandt et al., 2008). This behaviour leads to the winner of these encounters becoming dominant and the loser becoming subordinate. The subordinate crayfish alters its behaviour by avoiding contact with the dominant crayfish. This stereotyped behaviour is also demonstrated when crayfish are placed in a reflective

environment; a dominant crayfish approaches its reflection, and a subordinate crayfish avoids it. This thesis suggests that this behaviour is learned.

Learning is defined as a modification of behaviour as a result of experience (Purves et al., 1998) and a number of examples are exhibited by crayfish. Learning takes place during fighting and results in a change of behaviour. Crayfish that have won an agonistic encounter are more likely to win subsequent encounters (winner effect), while crayfish that have lost an agonistic encounter are more likely to lose subsequent encounters (Hsu & Wolf, 1999). Herberholz et al. (2001) showed that both medial giant (MG) and non-giant (NG) mediated tail flips become more excitable in subordinate crayfish once dominance has been decided. These neural pathways control different types of tail flips, which are performed more frequently by losing crayfish. Both of these examples clearly show that behaviour changes as a result of fighting and, thus, demonstrate that crayfish are learning through fighting. My earlier findings (May & Mercier, 2006; 2007) showed that fighting and acquisition of dominance ranks also alter behavioural responses to reflection. In the present study, consolidation appears to play an important role in both acquiescence to subordinate rank (as indicated by a decrease in aggression index) and in the appearance of "subordinate" responses to reflection. When given a break between fight trials, subordinate crayfish may be consolidating memory and learning their subordinate status more quickly than if no time to consolidate had been provided. It was previously thought that subordinate status in crayfish was obtained once the crayfish began avoiding its opponent (Yeh et al., 1997). The present thesis suggests that it may take longer to learn or develop a subordinate status than was previously thought. The correlation between responses to reflection and dominance status may be an

indication of the extent to which subordinate have learned their rank and have acquiesced to their losing status.

Often researchers will pair up crayfish for 30 minutes and determine which is dominant and which is subordinate, following analysis of wins and losses. This is commonly followed by pairing (housing together) the crayfish for some time, usually two weeks (Hazlett et al., 1975; Edwards et al., 2002; Song et al., 2006). Occasionally a rank reversal will occur during this time. That is, the crayfish that was determined to be dominant following initial pairing will now be subordinate and vice versa. This calls into question how long it actually takes to determine social status. It is possible that, after a 30 minute fight, one crayfish is simply the winner while the other is the loser, and that dominant/subordinate status may involve more complex behavioural changes that take longer to develop. Memory consolidation may play a role here. Previous work showed that when a delay is provided between fights, increasing the delay time decreased the probability that the winning crayfish would initiate a new fight (Bergman, et al., 2003). The effect that this delay has on the losing crayfish was not expressly investigated. Future experiments examining the role of learning and memory consolidation on the loser effect will expand our knowledge in this area.

The data reported here indicate that the dominant crayfish's response to reflection may be the "default" response, while the subordinate crayfish's response is altered by its status. Previous reports by this lab suggested this because both dominant and subordinate crayfish paired for 30 minutes responded to reflection similarly. A divergence in behaviour was only observed after three days of pairing (May & Mercier, 2007). The present thesis further supports this hypothesis and brings about additional

questions. How long does subordinate status last? How long does it take to extinguish subordinate status? The answers to these questions are important to further understand social status in animals. Combined with the research reported here, these answers will have practical implications for future social rank experiments and, potentially, for rearing and maintenance of crustacean species in research labs and in the food industry. Understanding the mechanisms underlying aggression could have far reaching implications for several issues, including social problems in humans.

Socialization Alters Responses of Fruit Flies to Reflection

This thesis has also shown for the first time that fruit flies are attracted to their reflection and that fruit flies that have been socialized respond to their reflection differently than those that were reared in isolation. The first ethogram of responses to reflection in fruit flies has been reported here.

Fruit flies have been an ideal model system for studying the effects of genes on behaviour. Until recently, such research has focused on the deficits or alterations in behaviour that are a result of changes in gene expression. In the past several years, however, researchers have begun to study the behaviour of wild-type *D. melanogaster*, in the hopes that some of the elusive questions regarding invertebrate behaviour can be answered with the fruit fly (Certel et al., 2007; Fernandez & Kravitz, 2013). Specifically, the mechanisms underlying dominance rank formation previously studied in crustaceans are now being examined in fruit flies. Like crayfish and lobster, fruit flies also engage in agonistic encounters and a dominance hierarchy is formed. While the fighting strategies

and behaviours differ, many of the dynamics and behaviours that change due to dominance status remain the same. Using this same strategy, to further understand how social experience influences responses to reflection, I chose the fruit fly as a model system.

The present thesis has shown that both male and female fruit flies prefer a reflective environment compared to a non-reflective environment. Further investigation showed that male fruit flies perform both grooming and courtship-like behaviours while in a reflective environment. The type of behaviours performed depended on how the flies were reared. Male fruit flies that had been reared in isolation performed more rear leg grooming in response to reflection, and the data showed a trend suggesting that these flies might also perform more courtship behaviours (wing vibration and proboscis extension) in response to reflection. Males reared in socialized conditions, however, did not show such tendencies. These observations suggested that isolated males might perceive their mirror image as a female. Being reared in isolation, they had never seen, smelled or touched another fruit fly, which means that they were naïve to the physical and chemical differences between male and female flies. An alternative explanation is that the mirror image was perceived to be male and the flies might choose to court it. The lack of statistical significance for effects of reflection on courtship-like behaviours suggested that the influence of visual inputs alone might not reach a threshold necessary for reflection to elicit courtship-related behaviours reliably. Further experiments that combined the application of pheromones with reflection were performed to help elucidate the results.

The cuticular hydrocarbon pheromones 11-cis vaccenyl acetate (cVA) and 7,11 heptacosadiene (7,11-HD) were both used to investigate the role of sex pheromones on

responses to reflection. The synthetic compounds were dissolved in ethanol and ethanol alone was applied to the control group for comparison. Behaviours performed by the ethanol groups were not significantly different in frequency than untreated flies previously examined for responses to reflection. While some differences were observed for individual behaviours, the total number of behaviours did not differ significantly for socialized fruit fly groups. Isolated fruit flies treated with either cVA or 7,11-HD exhibited a significantly reduced total number of behaviours compared to isolated flies treated with ethanol alone. Many pheromone-treated flies remained virtually stationary during the 20 min of recording and analysis of the video revealed that six fruit flies treated with 7,11-HD appeared to ejaculate on a wall surface. There are no reports of this occurring in the literature, but most pheromone studies involve mating and, thus, examined behaviour of males with a female fly present. If the substance was indeed ejaculate, a possible explanation is that the male fruit flies were attempting copulation with the mirror image. Future studies should take this observation into account and should confirm the identity of the substance left behind by some male flies.

The purpose of applying pheromones to the flies was to examine how an added sensory input (chemosensory) would influence responses to reflection. The doses used were found to have a maximum effect in other studies but may have been too high in the present study. A dose-response curve should be completed prior to further experiments.

Overall, the studies described in this thesis demonstrate that both crayfish and fruit flies are attracted to reflection, and that such attraction depends upon their interactions with conspecifics. Crayfish are attracted to reflection if they are socially dominant, possessing a status or behavioural state that develop through agonistic

interactions with another crayfish. Subordinate crayfish are not attracted to reflection and their social rank develops in a manner that suggests learning and memory. In fruit flies, attraction to reflection appears to depend on social interactions in a different way, since flies reared in isolation prefer a mirror but flies reared in social conditions do not. The reasons behind such apparent species differences are not clear, but it is worth noting that the present work did not examine effects of fighting on responses to reflection in fruit flies. Such studies should be pursued to determine how important the role of visual inputs may be in mediating conspecific recognition and agonistic interactions in arthropods.

In conclusion, the present thesis has added to our knowledge of responses to reflection in two invertebrate species and how social experience can alter those responses.

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APPENDIX 1

Analysis of Crayfish Fighting Behaviours

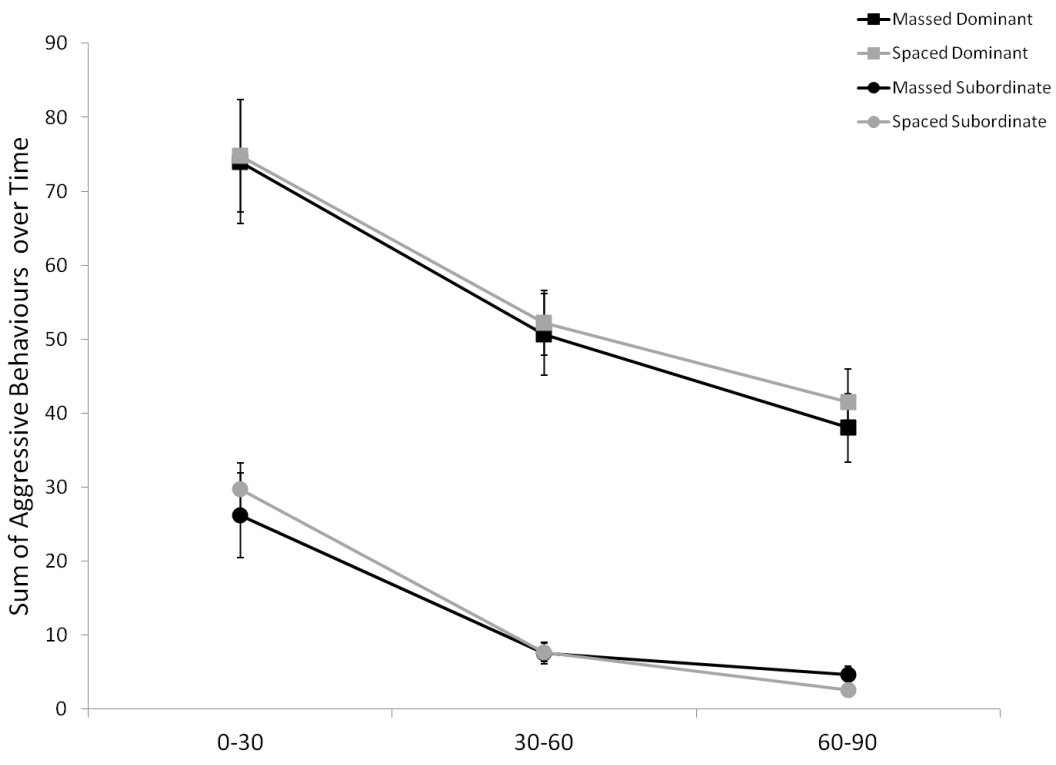


Figure A1: The sum of aggressive behaviours performed by each crayfish group over time in minutes. Aggressive behaviours include initiate, approach, push, pull, flip, strike and grasp. The number of aggressive behaviours performed declined over time for each group. There is no significant difference between the massed and spaced dominant crayfish or the massed and spaced subordinates for any time period.

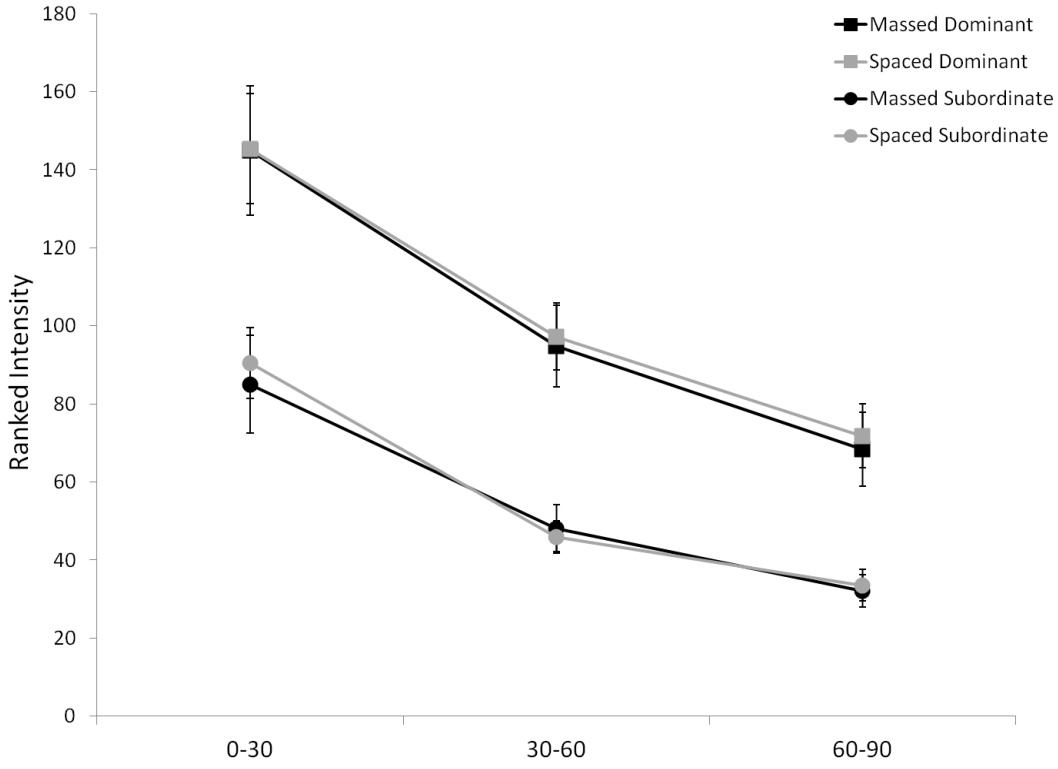


Figure A2: The ranked intensity of fighting behaviour for each crayfish group over time in minutes. Each behaviour, with its rank in brackets, used to calculate the intensity if listed here: initiate (2), approach (2), push (2), pull (3), retreat (1), avoid (1), tailflip (1), strike (2) and grasp (2). The intensity of fighting decreased over time for each crayfish group. There was no difference between massed and spaced dominants or massed and spaced subordinates for any of the time periods.

APPENDIX II

Histogram of Proboscis Extensions Performed by Male Fruit Flies

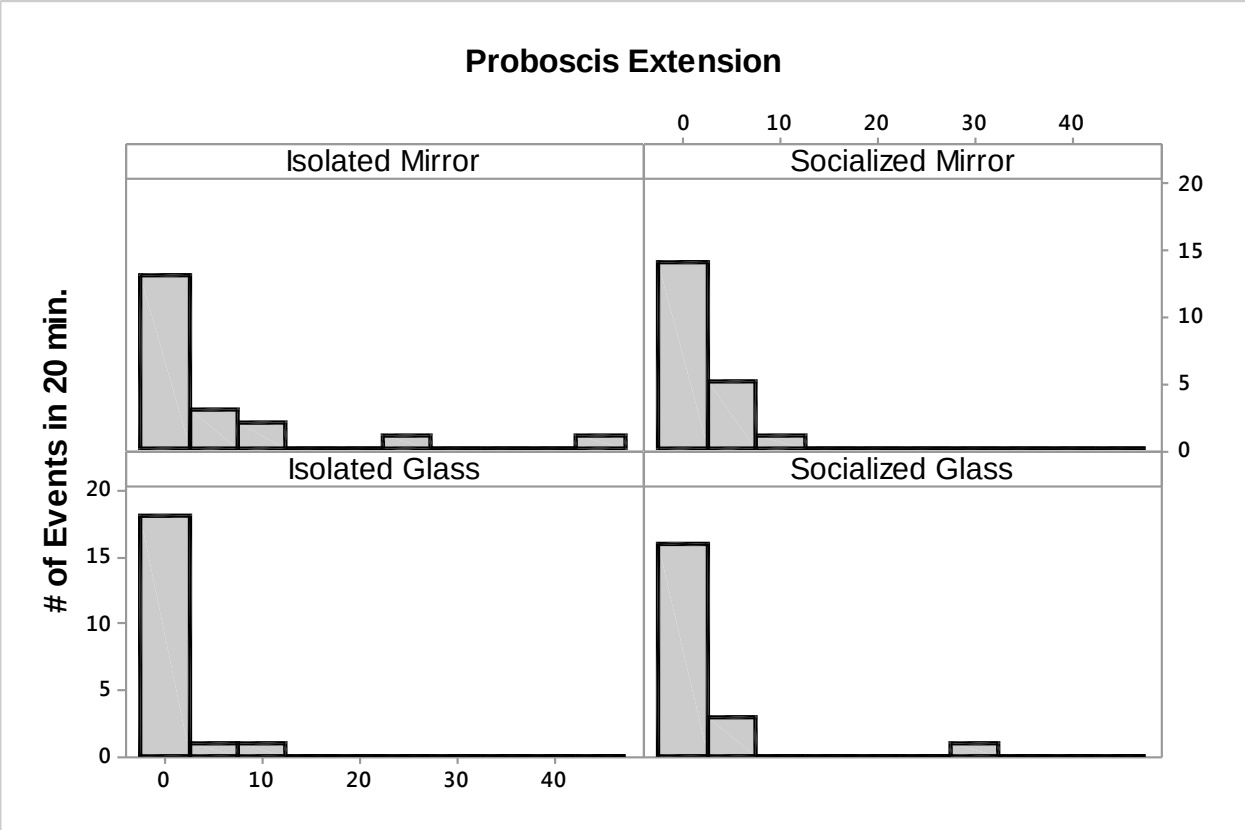
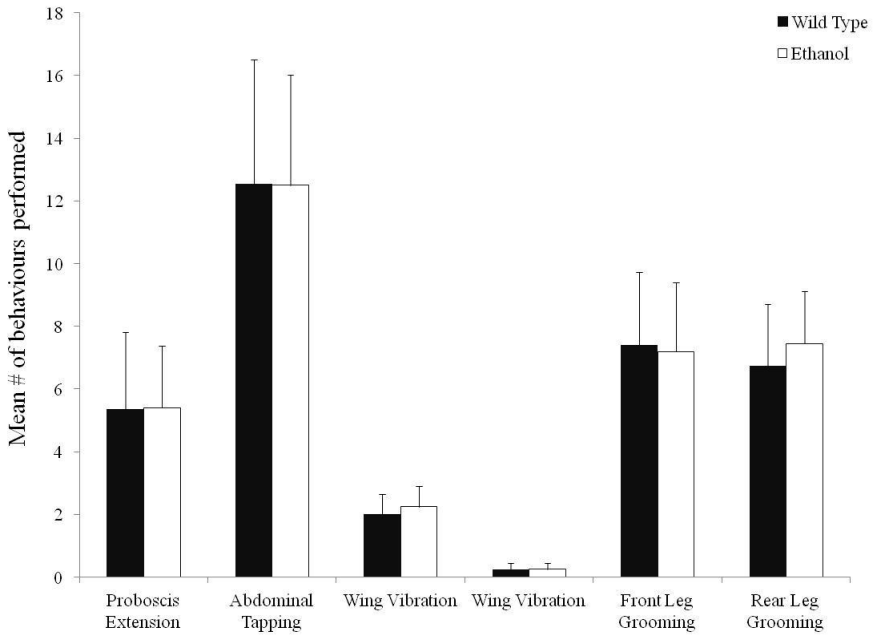


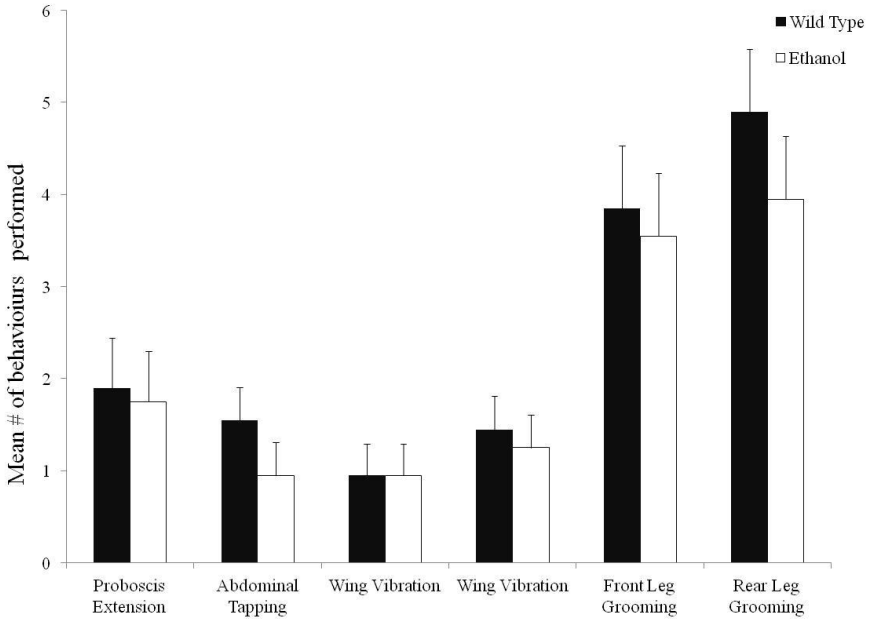
Figure A3: Histogram of proboscis extensions performed by male fruit flies. The x axis represents the number of behaviours performed (in bins of 10) and the y axis represents the number of fruit flies that performed that frequency. The proboscis extensions performed by isolated males on the mirrored side of the chamber were highly variable, with one fruit fly performing 46 events in 20 min.

APPENDIX III

Comparison of Responses to Reflection between Wild Type and Ethanol Treated Fruit Flies



a)



b) Figure A4. A comparison of responses to reflection between wild type male fruit flies and wild type male fruit flies treated with ethanol. a) There are no significant differences in responses between wild type flies reared in isolation and wild type isolated males treated with ethanol for each of the behaviours examines. b) There are no significant differences in the responses of socialized wild type male fruit flies and socialized wild type males treated with ethanol. Each of the behaviours shown here were performed on the reflective side of the observation chamber that had two walls comprised of mirrors and the other two comprised of glass. These figures demonstrate that ethanol had no effect on fruit fly behaviour.